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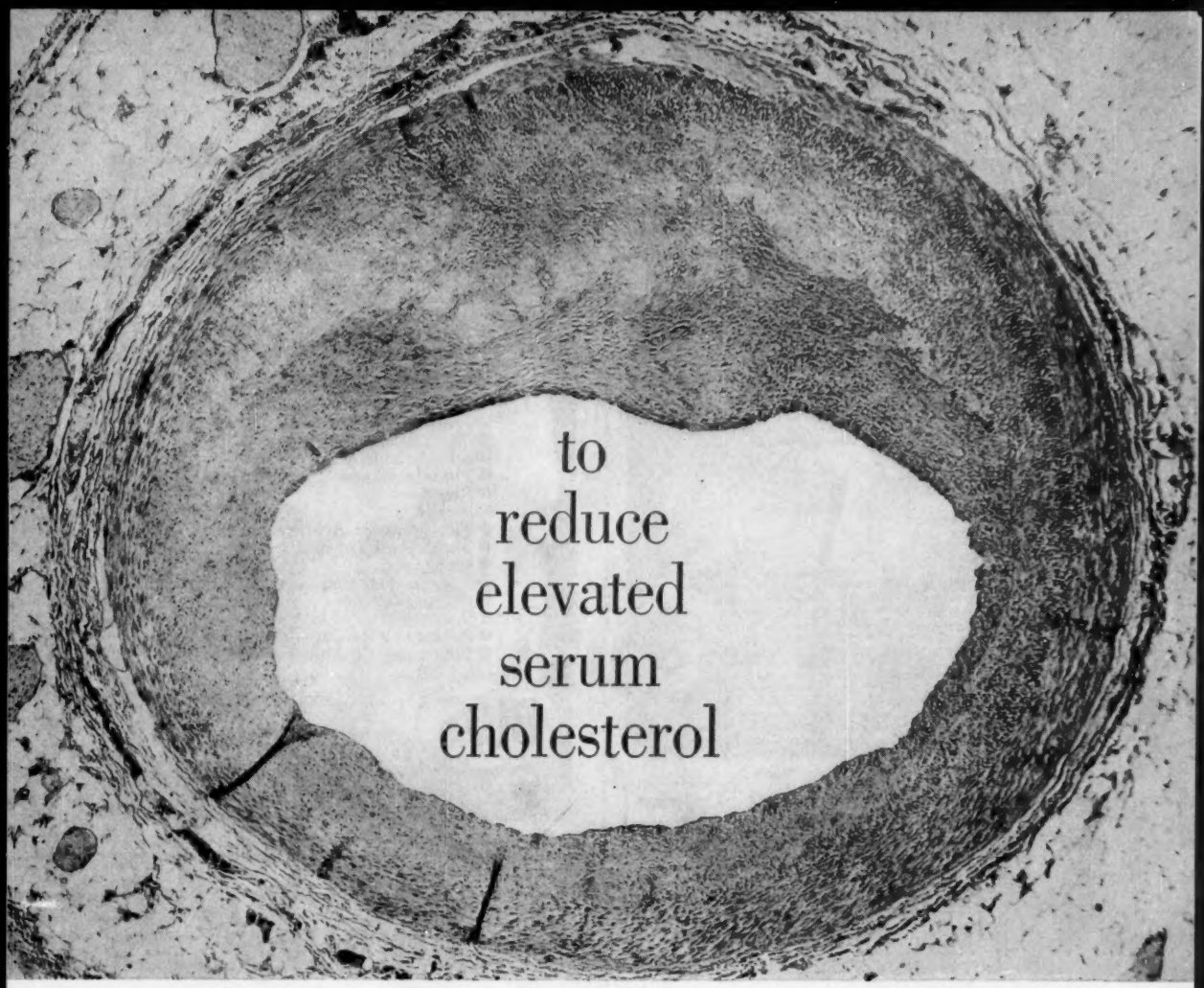

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Editorial

Etiologie, pathologie et pathogénie des épilepsies du lobe temporal. H. GASTAUT.

Original Articles

Introduction à la théorie mathématique de la physiologie hépatique. P. VENDRYES.

Phénomènes électrocorticaux provoqués chez l'animal par la stimulation d'afférences somesthésiques. R. MARTY, J. SCHERRER et F. CONTAMIN.

Les échanges de sodium durant l'hypothermie. Etude expérimentale à l'aide du radio-sodium chez le lapin. F. MOREL et A. COMBRISSON.

Activité de certaines protéines de la coagulation dans le plasma et les urines au cours du syndrôme néphrotique. H. YATZIDIS et G. RICHET.

Facteurs de l'hyperaldolasémie au cours des myopathies. F. SCHAPIRA, J. DEMOS, G. SCHAPIRA et J. C. DREYFUS.

Technical Note

Technique de recherche des auto-hémolysines immunologiques. J. DAUSSET, J. COLOMBANI et J. EVELIN.

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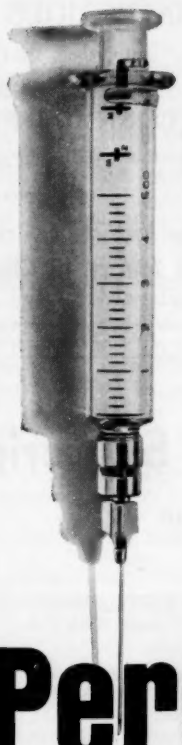

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1. Henstell, H. H., and Feinstein, M.: Am. J. Med. *22*:381 (March) 1957.

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Our Contribution

The 1956 Nobel Prize in Medicine and Physiology was awarded jointly to Doctors André Cournand,[2] Werner Forssmann and Dickinson Richards for their work in cardiac catheterization. In his acceptance lecture, Dr. Richards[3] stated it took ten years to assemble a comprehensive group of methods for the analysis of cardiopulmonary function, which could be applied not only to the normal but also to patients acutely ill. Among the techniques used was Gregerson's blood volume method using Evans Blue dye. Although not stated in the lecture, Warner-Chilcott supplied Evans Blue Warner-Chilcott in quantitative amounts in sealed ampuls requiring no precalibration of the syringe. Many of the techniques and materials adopted by Cournand, Forssmann, Richards and others are being further developed by circulatory system investigators. Chapman's recent publication on the use of large doses of Evans Blue, introduced into the heart for diagnosing intracardiac and aortic pulmonary shunts,[4] adds to the reports by earlier workers in this field. Evans Blue dye curves, oximetrically recorded, will so reliably identify the site and type of shunts that routine use of Evans Blue is urged for all cardiac catheterizations.

2. Cournand, A. F.: Am. Heart J. *54*:172 (Aug.) 1957.
3. Richards, D. W.: Am. Heart J. *54*:161 (Aug.) 1957.
4. Chapman, C. B., *et al.*: Am. Heart J. *53*:519 (April) 1957.

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5. Sie, Hsien-Gieh, and Fishman, W. H.: J. Biol. Chem. *225*:453 (March) 1957.

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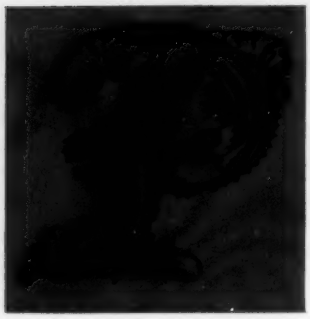

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**_Ibid._, p. 600.

THE EFFECT OF INSULIN ON BLOOD LEVELS OF INFUSED PENTOSES IN MAN

By STANTON SEGAL, JAMES B. WYNGAARDEN,[1] AND JOSEPH FOLEY

(From the National Institute of Arthritis and Metabolic Diseases, National Institutes of Health, Public Health Service, U. S. Department of Health, Education and Welfare, Bethesda, Md.)

(Submitted for publication December 17, 1956; accepted June 6, 1957)

In 1931 Wierzuchowski (1) showed an influence of insulin on galactose tolerance of dogs. More recently, Levine, Goldstein, Huddlestun, Klein, and Henry (2, 3) demonstrated that insulin lowers the blood levels of D-galactose, D-xylose, and L-arabinose but not of D-arabinose or D-mannose, following their infusion into the eviscerated nephrectomized dog. The volume of distribution of the responsive sugars was increased from 45 to 70 per cent of body weight as a result of insulin administration. It was suggested that the insulin response represented a facilitation of transport of the sugar across the cell membrane into intracellular fluid. In all the responsive sugars the stereochemical structure of carbon atoms 1 to 3 was like that of D-glucose, and these authors suggested that these structural characteristics determined the insulin responsiveness of a sugar.

Confirmatory evidence has appeared that insulin affects transport of D-galactose, D-xylose and L-arabinose across the cell membrane in the rat diaphragm (4–6), eviscerate rat (7), perfused rat heart (8) and intact cat (9). However, slow penetration of a number of sugars into muscle of nephrectomized and eviscerated rats has now also been demonstrated, and this slow penetration is increased by insulin for D-lyxose, D-ribose, D-mannose, D-fructose and D-fucose, as well as for D-xylose, D-galactose and L-arabinose (10, 11). Although these results indicate that the concept of structural specificity in carbon atoms 1 to 3 for insulin responsiveness has proved to be too limited in scope, the lack of an effect of insulin upon intracellular entry of sorbitol, mannitol and raffinose is strong evidence that the action of insulin is not simply an acceleration of a process of physical diffusion (12).

No reports have been noted demonstrating an effect of insulin on a sugar other than glucose in man. This paper reports studies on the effect of insulin on blood levels of infused pentoses in man. Pentoses were selected because their responses to insulin in animals have been well described, they are readily available, and following intravenous infusion they disappear from blood relatively slowly. This latter property permitted an evaluation of the effect of insulin upon blood levels and disappear-

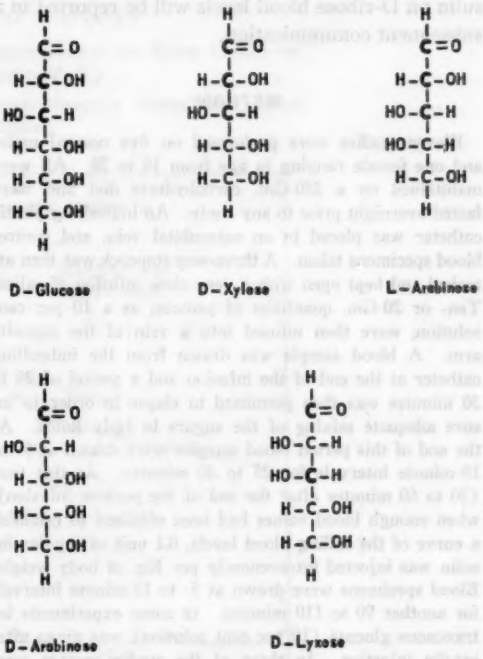

FIG. 1. THE STEREOCHEMICAL CONFIGURATION OF THE PENTOSES USED IN THE PRESENT STUDY

The carbons in bold type indicate those atoms whose configuration is similar to that of glucose in the first three atoms.

[1] Present address: Department of Medicine, Duke University School of Medicine, Durham, N. C.

ance rates, an appraisal which could not be made with galactose (13) or fructose (14), which disappear from blood even more rapidly than does glucose (15). In the present studies, we have investigated the influence of insulin upon the rate of pentose disappearance from blood, and upon the urinary excretion of pentose.

The stereochemical configuration of the sugars used in the present experiment is shown in Figure 1. Although these sugars exist in solution in a more complex pyranose ring structure, the simplified formulas shown here demonstrate certain differences in structure. D-xylose and L-arabinose have structures exactly like glucose in the first three carbon atoms. D-arabinose differs in spatial projection of hydrogen and hydroxl groups on carbons 2 and 3, and D-lyxose on carbon 2 alone. D-lyxose is the pentose whose structure in carbon atoms 1 to 4 resembles that of the hexose D-mannose. Because of the unusual kinetics of disappearance of D-ribose from blood, the effects of insulin on D-ribose blood levels will be reported in a subsequent communication.

METHODS

Eleven studies were performed on five normal males and one female ranging in age from 18 to 28. All were maintained on a 250-Gm. carbohydrate diet and were fasted overnight prior to any study. An indwelling plastic catheter was placed in an antecubital vein, and control blood specimens taken. A three-way stopcock was then attached and kept open with a very slow infusion of saline. Ten- or 20-Gm. quantities of pentose, as a 10 per cent solution, were then infused into a vein of the opposite arm. A blood sample was drawn from the indwelling catheter at the end of the infusion and a period of 25 to 30 minutes was then permitted to elapse in order to insure adequate mixing of the sugars in body fluids. At the end of this period blood samples were drawn at 5- or 10-minute intervals for 25 to 30 minutes. At this time (50 to 60 minutes after the end of the pentose infusion), when enough blood values had been obtained to establish a curve of the falling blood levels, 0.1 unit of regular insulin was injected intravenously per Kg. of body weight. Blood specimens were drawn at 5- to 15-minute intervals for another 90 to 110 minutes. In some experiments intravenous glucose (10 per cent solution) was given after insulin injection. In three of the studies sugars were infused at a constant rate after administration of a priming dose and the effect of insulin on the constant pentose level obtained was evaluated.

Urine was collected at various intervals over a 24-hour period.

TABLE I

Blood pentose levels after infusion

Subject	Pentose	Amount	Time of infusion	Blood pentose in mg. % at indicated time (minutes) after infusion																		Insulin		I.V. glucose	
				0	25	30	35	40	45	50	55	60	70	75	80	90	100	110	120	130	140	150	160	Amount	Time	Amount	Time
		Gm.	*min.*																						*min.*	*Gm.*	*min.*
J. D.	D-xylose	20	14		(20)† 53	47		43		38		32	22		16	15	15	14	13	10				8 U	51		
W. R.	D-xylose	20	8	87		61		51		52		47		42	(85) 32		(95) 25	21		(125) 21		(155) 15	(170) 13	7 U	65		
W. R.	D-xylose	20	24	(5) 75		62		(41) 55		53		50		32	(81) 31	(91) 24	(101) 21	19		15	14		14	7 U	61	25	73–107
D. B.	D-arabinose	*	120					32		31		31	31		31	32	31	31	31		(135) 22		21	8 U	70	50	80 p.o.
C. C.	D-arabinose	10	9	57	56	55	52	54	51		45		(65) 42	37		32		(105) 30	25		(135) 22		21	6 U	45	50	68–139
W. W.	L-arabinose	20	10	77	(20) 68	60		51		49		40	31	27	24	21		(105) 20	16		(135) 20	14		7 U	50	10 (ea.)	95 and 107 p.o.
S. B.	L-arabinose	10	18	41	30	29	27	26	23		(58) 18		(69) 14	(78) 12		(93) 10		(108) 9	(123) 7		(138) 5	(153) 4		7 U	48	25	62–107
C. C.	L-arabinose	20	22	61		52		46		41		37	(71) 33		(81) 23	(91) 22	(101) 18	(116) 15		(131) 11	(146) 9		(161) 3	6 U	61	50	78–144
W. R.	D-lyxose	20	7	118		77		68		55		50	(71) 46		(81) 40	(91) 36	(101) 32	(116) 29		(131) 23	(146) 22		(161) 21	7 U	61	50	84–133
D. B.	D-lyxose	*	130					32		33		31	31		30	27	27	26	26	27				8 U	70	50	80 p.o.

* Constant infusion studies 8 Gm. prime; 76 mg. per minute. † Figures in parentheses are the exact time in minutes for the given pentose level.

All of the pentoses initially employed were obtained from Dr. Hewitt G. Fletcher, Jr., of the Section on Carbohydrates, Laboratory of Chemistry of this Institute, and recrystallized before use. All had specific optical rotations corresponding with those of the pure sugars reported in the literature (16). Subsequently, D-xylose and L-arabinose were obtained from Pfanstiehl Laboratories, Incorporated, Waukegan, Illinois. The sugars were prepared for intravenous use by autoclaving as 10 per cent solutions, and were found sterile and pyrogen-free prior to use.

Blood glucose and pentose levels were determined by

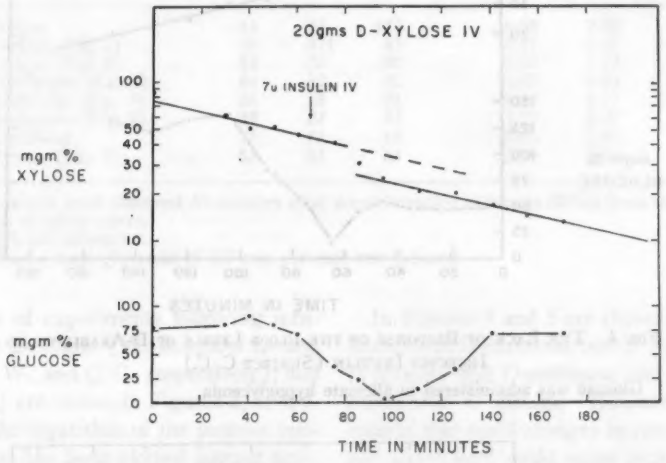

FIG. 2. THE EFFECT OF INSULIN ADMINISTRATION ON BLOOD LEVELS OF D-XYLOSE (SUBJECT W. R.)

Blood xylose levels are plotted hemilogarithmically versus time. Blood glucose values are plotted on normal ordinates.

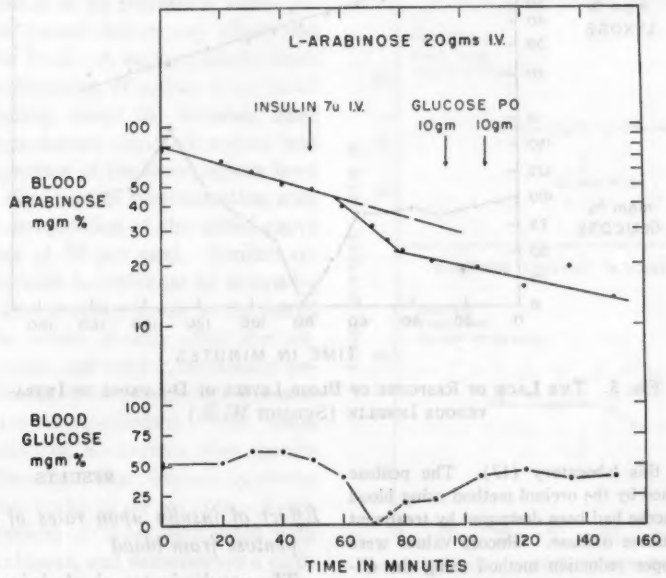

FIG. 3. THE EFFECT OF INSULIN ON THE BLOOD LEVELS OF L-ARABINOSE (SUBJECT W. W.)

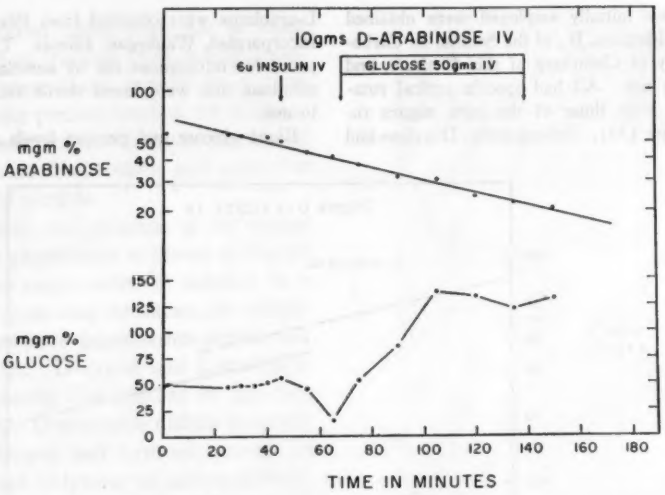

FIG. 4. THE LACK OF RESPONSE OF THE BLOOD LEVELS OF D-ARABINOSE TO INJECTED INSULIN (SUBJECT C. C.)

Glucose was administered to alleviate hypoglycemia.

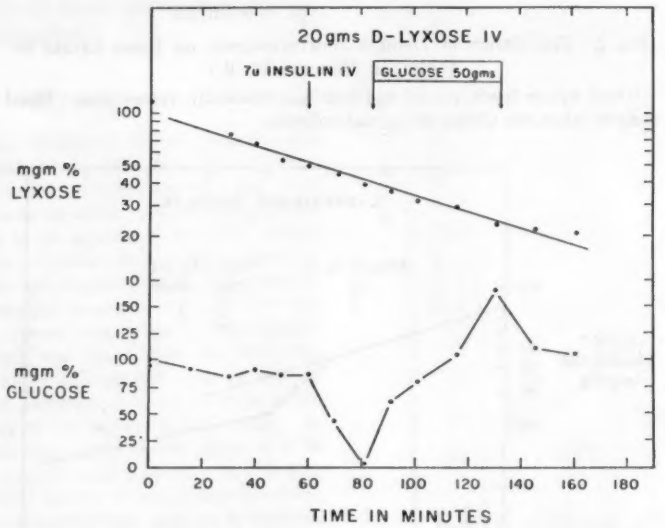

FIG. 5. THE LACK OF RESPONSE OF BLOOD LEVELS OF D-LYXOSE TO INTRAVENOUS INSULIN (SUBJECT W. R.)

methods devised in this laboratory (17). The pentose values were determined by the orcinol method using blood filtrates in which glucose had been destroyed by treatment with the enzyme glucose oxidase. Glucose values were determined by a copper reduction method using the difference between values obtained before and after incubation with the enzyme.

RESULTS

Effect of insulin upon rates of disappearance of pentose from blood

The results in ten single injection and constant infusion experiments performed are tabulated in

TABLE II

Biologic half-time and rates of disappearance of infused pentoses

Subject	Sugar	Biologic half-time (T ½) in minutes			Per cent disappearance/min. (K) × 100			% Fall in pentose level*
		Initial	30 min. post insulin	Final	Initial	30 min. post insulin	Final	
J. D.	D-xylose	64	24	122	1.08	2.89	0.57	40
W. R.	D-xylose (Fig. 2)	86	*27*†	87	0.81	*2.57*	0.80	31
W. R.	D-xylose (Fig. 8)	85	*29*	80	0.82	*2.39*	0.87	38
C. C.	D-arabinose (Fig. 4)	82	82	82	0.85	0.85	0.85	0
W. W.	L-arabinose (Fig. 3)	68	25	91	1.03	2.77	0.76	34
S. B.	L-arabinose (Fig. 9)	58	*28*	47	1.19	*2.47*	1.47	26
C. C.	L-arabinose	74	*19*	48	0.94	*3.64*	1.44‡	20
W. R.	D-lyxose (Fig. 5)	63	63	63	1.10	1.10	1.10	0

* Percentage by which level observed 30 minutes after administration of insulin differs from that calculated at this time by extrapolation of initial curve.

† Figures in italics are estimated.

‡ During this phase a hyperglycemia of 235 mg. per cent was induced.

Table I. Graphs of experiments following infusion of 20 Gm. of D-xylose (W. R.), and L- and D-arabinose (W. W., and C. C., respectively), and D-lyxose (W. R.) are shown in Figures 2, 3, 4, 5. In these figures the logarithm of the pentose concentration in blood has been plotted against time. In Figure 2 the portion of the curve of D-xylose concentrations obtained from 0 to 75 minutes after infusion demonstrates the hemilogarithmic linear disappearance curve of this pentose from blood. After insulin injection at 65 minutes a latent period of 10 minutes passed before any effect was noted in the xylose level. A marked acceleration in the rate of disappearance of xylose from blood then occurred, lasting about 20 minutes, after which a new disappearance curve of xylose was established. Comparison of the blood xylose level found 30 minutes after insulin administration with that obtained by extrapolation of the initial curve reveals a difference of 30 per cent. Similar results were obtained with L-arabinose as shown by Figure 3. Here, also, a sharp break in the initial disappearance rate occurs shortly after the administration of insulin, and after a 30-minute period of rapid decline in blood pentose level, a new disappearance curve is established. The blood arabinose level measured 30 minutes after insulin administration differs from that obtained by extrapolation of the initial curve by 34 per cent. These experiments are typical of those performed with D-xylose and L-arabinose, and demonstrate a positive response of these sugars to injected insulin (Table II).

In Figures 4 and 5 are shown the responses obtained with D-arabinose and D-lyxose, respectively. It appears that D-arabinose and D-lyxose are unresponsive to insulin. However, the possibility existed that small changes in rate of disappearance and blood level could occur in the 30-minute post insulin period without being detected in this type of experiment. Therefore, two experiments were

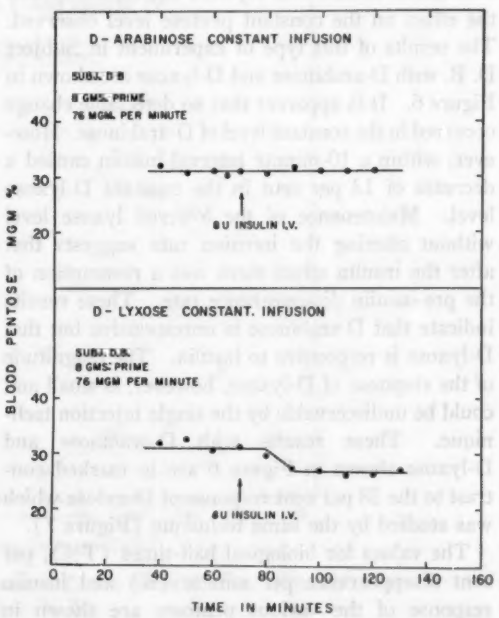

FIG. 6. THE EFFECT OF INSULIN ON CONSTANT BLOOD LEVELS OF D-ARABINOSE AND D-LYXOSE

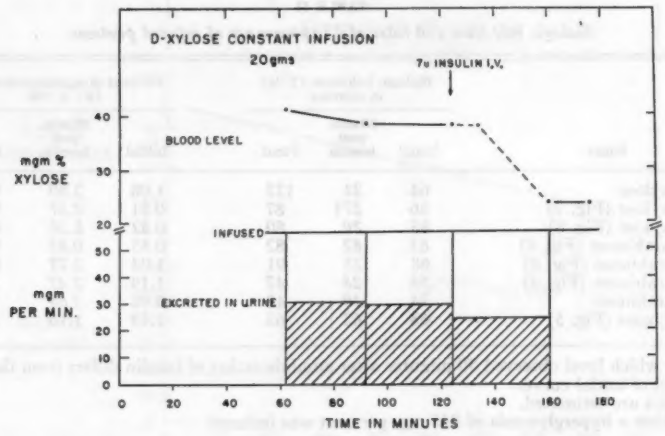

FIG. 7. THE EFFECT OF INSULIN ON BLOOD LEVELS AND URINARY EXCRETION OF D-XYLOSE (SUBJECT W. R.)

The fall in blood levels of D-xylose resulting from insulin administration cannot be attributed to an increase in urinary excretion.

carried out employing a constant infusion technique. After a priming dose the pentose was infused at a slow constant rate by means of a Bowman pump. When a constant blood level had been established insulin was injected intravenously and the effect on the constant pentose level observed. The results of this type of experiment in Subject D. B. with D-arabinose and D-lyxose are shown in Figure 6. It is apparent that no detectable change occurred in the constant level of D-arabinose. However, within a 10-minute interval insulin caused a decrease of 13 per cent in the constant D-lyxose level. Maintenance of the lowered lyxose level without altering the infusion rate suggests that after the insulin effect there was a resumption of the pre-insulin disappearance rate. These results indicate that D-arabinose is unresponsive but that D-lyxose is responsive to insulin. The magnitude of the response of D-lyxose, however, is small and could be undiscernable by the single injection technique. These results with D-arabinose and D-lyxose shown in Figure 6 are in marked contrast to the 38 per cent response of D-xylose which was studied by the same technique (Figure 7).

The values for biological half-times ($T\frac{1}{2}$), per cent disappearance per minute (K) and insulin response of the various pentoses are shown in Table II. In the D-xylose experiments in W. R. (Figures 2 and 8) the initial and final xylose disappearance rates are almost identical despite the interposition of a half-hour period of rapid disappearance. The D-xylose study in J. D. reveals a final disappearance rate which is much slower than the initial. Some variability of initial and final rates is seen in the L-arabinose studies. W. W. (Figure 3) demonstrates a slower final rate, whereas S. B. (Figure 9) and C. C. (Figure 4) show slightly faster rates.

In the L-arabinose study of W. W. (Figure 3) enough points were obtained in the half-hour after insulin to permit a calculation of values for the rapid disappearance phase. The $T\frac{1}{2}$ is 25 minutes for this curve, indicating an almost three-fold increase in disappearance rate in the short period following insulin administration. The same calculation can be made for D-xylose from the data on J. D. and again the rate increase is nearly three-fold. Not enough points were obtained in the 30-minute period post insulin in the other studies with D-xylose and L-arabinose to yield an accurate curve. However, if one estimates the minimal rate of disappearance in this period from a theoretical line connecting the last value of the initial curve with the first value of the final curve, one finds rates very similar to those actually determined in other studies.

Table II also indicates the degrees of response of the pentoses to insulin as measured by the single

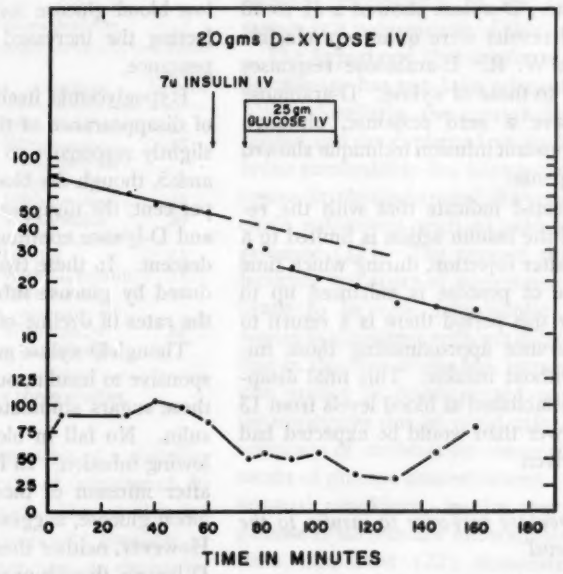

FIG. 8. THE FAILURE OF INTRAVENOUS GLUCOSE ADMINISTRATION TO ABOLISH THE EFFECT OF INSULIN ON DISAPPEARANCE OF D-XYLOSE FROM BLOOD (SUBJECT W. R.)

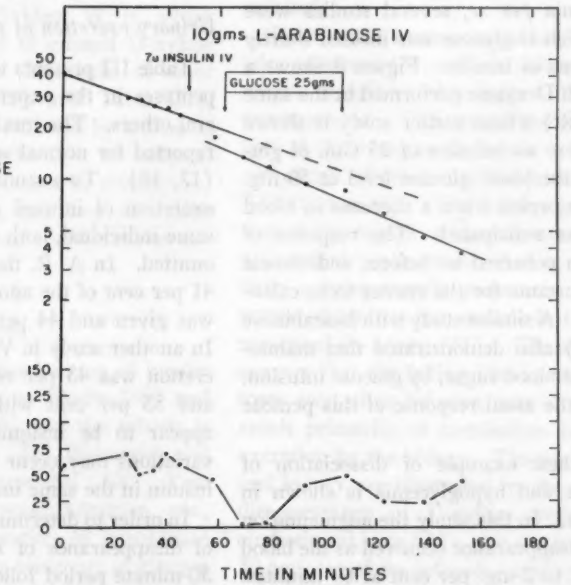

FIG. 9. THE DISSOCIATION OF THE INSULIN EFFECT FROM HYPOGLYCEMIA *Per Se* (SUBJECT S. B.)

The insulin response of the pentose has terminated though blood glucose values remain at low levels.

injection technique. D-xylose showed a 31 to 40 per cent fall, and results were quite reproducible in two studies in W. R. L-arabinose responses were very similar to those of xylose. D-arabinose and D-lyxose have a zero response, although D-lyxose by the constant infusion technique showed a 13 per cent response.

The data presented indicate that with the responsive pentoses the insulin action is limited to a half-hour period after injection, during which time the disappearance of pentose is enhanced up to three-fold. After this period there is a return to rates of disappearance approximating those initially observed without insulin. This final disappearance rate is established at blood levels from 13 to 40 per cent lower than would be expected had no insulin been given.

Relationship of pentose response to insulin to the blood glucose level

The response of D-xylose and L-arabinose as shown in Figure 2 and Figure 3 was observed in the presence of very low blood glucose values. In order to dissociate the pentose response to insulin from hypoglycemia *per se,* several studies were performed in which D-glucose was infused shortly after the injections of insulin. Figure 8 shows a second study with D-xylose performed in the same individual (W. R.) whose earlier study is shown in Figure 2. Here an infusion of 25 Gm. of glucose maintained the blood glucose level at 50 mg. per cent over the period when a decrease in blood xylose levels was anticipated. The response of xylose to insulin occurred as before, and almost identical rate constants for the curves were calculated (Table II). A similar study with L-arabinose (C. C., Table I) also demonstrated that maintenance of a normal blood sugar, by glucose infusion, did not prevent the usual response of this pentose to insulin.

Perhaps the best example of dissociation of pentose response and hypoglycemia is shown in Figure 9 (S. B.). In this study the augmentation of L-arabinose disappearance occurred as the blood glucose level fell to 2 mg. per cent at 69 minutes. However, despite the continuation of hypoglycemia, the slope of the disappearance curve of L-arabinose returned to a value similar to that prior to insulin administration. This indicates that a low blood glucose level *per se* is not a factor affecting the increased rate of L-arabinose disappearance.

Hypoglycemia itself does not influence the rate of disappearance of those sugars unresponsive or slightly responsive to insulin. Thus, in Figures 4 and 5, though the blood glucose is less than 5 mg. per cent, the disappearance curves of D-arabinose and D-lyxose continue in a smooth uninterrupted descent. In these two studies hyperglycemia produced by glucose infusion was without effect on the rates of decline of pentose levels.

Though D-xylose and L-arabinose are readily responsive to insulin, our results do not suggest that these sugars stimulate the pancreas to secrete insulin. No fall in blood sugar was observed following infusion. In fact, as previously seen (17), after infusion of these sugars there is a rise in blood glucose, suggesting they may be glucogenic. However, neither these sugars nor D-arabinose or D-lyxose, though present at levels of 30 to 40 mg. per cent, were able to prevent the hypoglycemic symptoms experienced by those individuals with low blood glucose levels.

Urinary excretion of pentoses

Table III presents the total urinary excretion of pentoses in the experiments described, plus several others. The total excretions are in the range reported for normal subjects not receiving insulin (17, 18). Two studies are shown in which the excretion of infused sugar was measured in the same individual, both when insulin was given and omitted. In A. R. the excretion of D-xylose was 41 per cent of the administered dose when insulin was given and 44 per cent when it was omitted. In another study in W. W., total L-arabinose excretion was 43 per cent of the dose with insulin and 53 per cent without insulin. These would appear to be insignificant differences, for such variations may occur on repeated studies without insulin in the same individual.

In order to determine whether the increased rate of disappearance of D-xylose from blood in the 30-minute period following insulin administration could be explained by increased urinary excretion during that interval, the experiment shown in Figure 7 was performed. This figure presents data obtained during a constant infusion of

TABLE III

Urinary excretion of infused pentoses

Subject	Sugar infused	Conditions	% of dose excreted
W. R. (Fig. 2)	D-xylose	With insulin	42
W. R. (Fig. 8)	D-xylose	With insulin and glucose	55
A. R.*	D-xylose	With insulin	41
A. R.*	D-xylose	Without insulin	44
C. C. (Fig. 4)	D-arabinose	With insulin and glucose	56
W. W. (Fig. 3)	L-arabinose	Insulin	43
W. W.*	L-arabinose	Without insulin	53
S. B. (Fig. 9)	L-arabinose	With insulin and glucose	60
W. R. (Fig. 5)	D-lyxose	With insulin and glucose	55

* Blood levels not determined in these studies.

D-xylose. After the administration of a priming dose of 10 Gm., a constant rate of infusion of 56 mg. per minute was maintained. During this infusion the blood level was essentially constant at 38 mg. per cent. Urinary excretion, as shown by crosshatching, occurred at a rate of 30 mg. per minute, leaving 26 mg. per minute, or 46 per cent of the amount infused unaccounted for. These values obtained at equilibrium compare well with the overall results in this individual, W. R., which show that 42 to 55 per cent of infused D-xylose appeared in urine and with the results of other studies with this sugar [(17) and Table III]. The injection of insulin was followed by a drop of 38 per cent in blood pentose level in 30 minutes. During this time a slight decrease in urinary excretion was noted. Hence, this loss of xylose from blood after insulin injection could not be explained on a renal basis.

DISCUSSION

Reviews on the mechanism of action of insulin have recently been published by Stadie (19) and by Levine and Goldstein (20), and the subject is further discussed by deDuve (21), Helmreich and Cori (11), and Park and associates (10). Two hypotheses have received recent attention, *viz.*, that insulin accelerates the action of hexokinase and that insulin facilitates the transport of glucose into the cell. This latter hypothesis has recently been modified by Helmreich and Cori (11), yielding in effect a third concept, that insulin affects intracellular metabolism in such a way that increased permeability of the cell membrane to sugars is a consequence of this action.

The hypothesis that insulin acts at the level of hexokinase has not been adequately substantiated (19). Similarly, the concept that the insulin response can be explained as an increase in membrane permeability has been criticized, since an increase in physiochemical diffusion should not exclude substances such as sorbitol, mannitol and raffinose (12). The concept that insulin acts to facilitate transport of sugars across the cell membrane to increase intracellular concentration of sugars is consistent with available evidence, except that in the case of glucose, intracellular accumulation cannot be demonstrated under physiological circumstances due to rapid utilization and the inadequacy of methods for measuring minute increments of glucose concentrations. However, under unusual conditions, insulin may also cause free glucose to accumulate intracellularly. Park, Bornstein, and Post (22) demonstrated in the rat diaphragm that when hexokinase was saturated by high glucose levels in the medium, or was inhibited by low temperature, insulin would indeed cause glucose from the medium to accumulate intracellularly.

That insulin causes an increase in free intracellular galactose (7) and L-arabinose (8) content of muscle as well as other sugars (10) has also been demonstrated. This would indicate that metabolism of these sugars in muscle is minimal or does not occur. Park found the latter to be true for L-arabinose (8). The Levine group found also that these sugars were not metabolized by the eviscerated nephrectomized dog, which is essentially a living muscle preparation. The slow entrance of pentose into muscle in the normal rat is measured in hours (11). These findings would suggest that the falling blood levels of these pentoses seen after infusion into intact man are the result primarily of metabolism by the liver and excretion by the kidney. The major roles of liver and kidney are illustrated by the findings of slow disappearance of D-xylose from the blood in cirrhotic subjects (17), and the excretion of about 50 per cent of the infused quantities of pentoses in urine by the subjects of the present study.

In the present study the administration of insulin caused an abrupt increase in the rates of disappearance of D-xylose and L-arabinose and

D-lyxose from the blood stream, lasting up to 30 minutes, after which time the disappearance rates abruptly decreased and in most studies approximated the initial rates of disappearance as determined before injection of insulin. This response to insulin was not dependent upon development of hypoglycemia, and reached completion despite evidence of continued action of insulin on glucose. From considerations of the dose of insulin injected, of recent evidence of disappearance rates and volumes of distribution of injected insulin (23), and of blood levels of insulin following injections of small amounts (24), it may be estimated that the blood insulin level was three to six times normal at a time when the pentose response was complete. It appears to us unlikely that an increase in rate of pentose metabolism caused by insulin as its primary action would be reflected in the type of response that was observed, namely the abrupt termination of the phase of rapid disappearance of pentose despite the presence of abundant insulin.

Bearn, Billing, and Sherlock (25) have claimed that insulin causes a rapid uptake of glucose by the liver in man. At times other investigators have purported to have shown a direct effect of insulin *in vitro* upon glucose uptake and glycogen synthesis of rat liver slices (21), but this subject is controversial and other studies suggest that insulin itself has no direct action on carbohydrate metabolism by the liver of the rat (26). However, in view of the findings of Bearn and collaborators, one must consider the possibility that insulin has caused a rapid uptake of D-xylose and L-arabinose by liver in the subjects of this report. There are, however, certain imposing arguments against this interpretation. First, similar responses were demonstrated by Levine and associates (20, 26) in dogs from whom livers had been removed. Second, the observations that metabolism of D-xylose is unimpaired in diabetics deprived of insulin (27), and that the rate of disappearance and urinary excretion of infused D-xylose are normal in diabetics off insulin (28), suggest that the hepatic system for handling this pentose is not insulin dependent. Third, Helmreich and Cori (11) have shown in the nephrectomized rat that the insulin effect on pentoses is clearly to increase the amount present in muscle and that the liver being present is not an interfering factor in studies of this type. Several studies have clearly shown that uptake of sugars by brain is not influenced by insulin (10). We favor the interpretation that insulin has acted in the present studies to increase the distribution of D-xylose and of L-arabinose and D-lyxose in the intracellular fluids of man primarily in muscle by facilitating their transfer across the cell membrane.

The glucose "space" has been estimated in intact man (29) and in the eviscerated rabbit (30) employing techniques with C^{14} glucose. Shreeve *et al.* (29) have found the glucose space to vary only minimally from normal in the human diabetic, and Drury and Wick (30) have shown that insulin does not increase the glucose space in their rabbit preparation. However, one would not necessarily anticipate that insulin would increase the glucose space even though it increased transport of glucose into the cell, for all glucose passing across the cell membrane may normally be rapidly phosphorylated by action of hexokinase. Although the determination of a volume of distribution of metabolized substance involves some error, we have estimated the pentose "space" to be 21 per cent of body weight (17). Assuming this volume, and assuming the changes of 20 to 40 per cent in blood levels of D-xylose and L-arabinose resulting from insulin administration are due to distribution changes, one can calculate the pentose space after a single intravenous injection of 0.1 unit of insulin per kilo of body weight to be about 32 per cent of body weight, or 1.5 times the initial value. This volume of distribution is somewhat lower than that reported by Levine, Goldstein, Huddlestun, and Klein (2) for the dog after insulin administration, but the proportional increase in volume of distribution caused by insulin is the same. The D-lyxose space may be calculated to have increased to only 24 per cent of body weight. The increases in volume of distribution calculated here correspond to those reported by Helmreich and Cori (11) in the nephrectomized rat. These investigators report no significant effect of insulin on D-arabinose volume of distribution. It should be mentioned that since the insulin effect is primarily to increase pentose concentration in muscle (10, 11) calculation of changes in volume of distribution from changes in blood levels will give results less than the average total body water.

A final point requiring comment is the relation-

ship of insulin responsiveness and structural configuration. That the configuration of carbon atoms 1 to 3 has some significance with respect to insulin responsiveness has been shown only in the eviscerated dog (3). In the rat (10, 11) and rabbit (31) corresponding specificity has not been demonstrated. In man it would appear that although the most responsive sugars, D-xylose and L-arabinose, are related to glucose in carbon atoms 1 to 3 and that D-arabinose, which differs from glucose in carbons 2 and 3, is unresponsive, D-lyxose differing in carbon 2 alone has a slight degree of responsiveness. Part of this discrepancy may be due to species differences and part may be a matter of rate of pentose transport as determined by the insulin doses given in the various studies. For example, Goldstein, Henry, Huddlestun, and Levine (3) gave a 5-unit insulin prime and a constant infusion of 1 unit per kilo per hour. Helmreich and Cori (11) gave 1 to 2 units per 100 Gm. The latter dose on a weight basis is one to two hundred times the dose used in the present study. In man a discernable response of D-xylose to insulin may be seen with as little as 0.05 unit per kilo (32). It would appear that man is more sensitive to insulin than the other animal species tested.

At the present time no general theory of sugar structure explains insulin responsiveness in all species. In the rabbit for example, glucosamine (33) and 2-deoxyglucose (34) both appear to be insulin responsive so that in this species the substituents at carbon 2 are not determining with respect to insulin responsiveness. However, although the membrane transport theory of insulin action cannot be considered proved, we believe this concept to be consistent with the greatest number of experimental observations.

SUMMARY

The insulin responsiveness of certain pentoses has been investigated in man. Blood levels of D-xylose, L-arabinose and D-lyxose are affected by insulin, whereas those of D-arabinose are not. The observed response consists of an abrupt lowering of the pentose level which appears to be maximal and complete after 30 minutes, at a time when plasma insulin values would be expected to be three to six times the fasting value. The response in man cannot be attributed to increased renal excretion. The results obtained suggest that insulin has affected the volumes of distribution of D-xylose, L-arabinose and D-lyxose in the body fluids of man.

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PHYSIOLOGICAL DISPOSITION AND METABOLIC FATE OF INFUSED PENTOSES IN MAN

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In recent years there has been an increasing interest in the role of pentoses in carbohydrate metabolism. It is now clearly established that a number of pentose phosphates occur as normal intermediates in the oxidative catabolism of glucose. Although many of the reactions involving the pentoses were first encountered in studies of bacterial or plant carbohydrate metabolism, there is now evidence that mammalian tissues possess many of the same biochemical pathways.

Normal human urine regularly contains measurable quantities of the aldopentoses xylose, arabinose and ribose (1, 2), and of the ketopentoses, D-ribulose and L-xylulose (2–4) in addition to small quantities of glucose, fructose, fucose, galactose, sucrose, lactose, mannoheptulose and sedoheptulose (1, 2, 5). Patients with essential pentosuria excrete gram quantities of the ketopentose, L-xylulose, independent of diet (6–8). Ingestion of foods high in pentosan content, especially fruits and grains, is associated with a type of pentosuria called alimentary pentosuria (9). The pentose excreted reflects that ingested, generally xylose or arabinose (1). In addition, there are poorly defined pentosurias thought to be toxic or drug-induced (10, 11) or associated with neurological disease (1).

A further point of great interest is that certain pentoses have been shown to be insulin responsive in various experimental animals. This responsiveness, interpreted by most workers as representing a facilitation of pentose transfer across cell membranes into intracellular fluid, was first thought to be restricted to those pentoses possessing a glucose-like stereochemical constitution of carbon atoms 1 to 3 (12). Recent studies have shown, however, that insulin responsiveness is not restricted to such sugars (13, 14).

Because of the increasing awareness of the biological importance of the pentoses, and because of the paucity of information regarding their metabolism in man, we have investigated certain phases of pentose metabolism in normal human subjects. This paper presents data on the physiological disposition of infused D-xylose, D-arabinose, L-arabinose, and D-lyxose in man, and observations on the effects of these pentoses on blood levels of glucose, lactate and pyruvate, and inorganic phosphorus. Data on the effects of insulin on blood levels of infused pentoses are presented in a companion paper (13). Studies have also been performed with D-ribose, but because the kinetics of its disposition, and the extent of its utilization, are quite different from the pentoses listed above, these data will be presented in a separate communication.

MATERIALS AND METHODS

D-xylose, D-arabinose, L-arabinose, and D-lyxose were obtained from Dr. Hewitt G. Fletcher, Jr., of the Section on Carbohydrates, Laboratory of Chemistry, of this Institute, or purchased from Pfanstiehl Chemical Company, Waukegan, Illinois. The purity of each sugar was checked by optical rotation (15), and when indicated the sugar was purified by recrystallization from water using alcohol and seeding to induce crystal formation (16). Ten per cent solutions, suitable for intravenous infusion, were prepared by the National Institutes of Health pharmacy and were tested and found sterile and nonpyrogenic prior to use. D-xylose-1-C^{14}, specific activity 0.67 μc. per mg., was purchased from the National Bureau of Standards.

The subjects of this study were normal male and female volunteers, ranging in age from 18 to 26 years. All subjects were maintained on diets containing at least 250 Gm. of carbohydrates daily, and were fasted overnight prior to any study.

At the start of a procedure, an indwelling No. 18 polythene needle was placed in an antecubital fossa vein, and attached to a three-way stopcock, through which normal saline was infused slowly. Two blood samples were then drawn, 10 minutes apart, for control chemical determinations. These and all subsequent samples were drawn through the indwelling needle without preliminary

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stasis. After control blood and urine samples were obtained, the sugar to be studied was infused in 10- to 20-Gm. quantities in the opposite arm over a 20-minute period. Thereafter, periodic blood samples were drawn for three hours, after which the subject was permitted to eat his normal meals. Fractional urine specimens were obtained for 24 hours, and preserved by freezing.

Analytical procedures

Glucose. Blood was collected in tubes containing fluoride and oxalate to prevent glycolysis and coagulation. Blood filtrates were prepared by the Somogyi procedure (17) and glucose was determined by the Nelson-Somogyi method (18, 19).

Pentose. In order to determine accurately the concentrations of glucose and of pentose or other hexose when glucose and a second sugar were present simultaneously in blood or urine, we have adapted an industrial method designed to determine true glucose in mixtures of sugars (20). This method employs glucose oxidase to oxidize glucose selectively. Glucose can be measured by titration of the product, gluconic acid (20), by measurement of oxygen consumption (21), or by change in reducing power of the mixture, as in the present paper. Furthermore, once glucose is destroyed, pentose can be determined by the standard orcinol reaction without glucose interference. Glucose oxidase is relatively specific for glucose (22), acting upon its D-β-glucopyranose form (23), converting it to D-gluconolactone, and generating peroxide in the reaction. Several commercial preparations of glucose oxidase are available, all containing mutarotase (which catalyzes the interconversion of α and β-glucopyranose) and catalase (which destroys the peroxide). In principle, the method used in these studies was similar to that developed by Froesch and Renold (5), but since the details of the procedure developed in our laboratory were quite different, it is presented here.

Procedure

(All glassware must be scrupulously cleaned with detergent followed by sulfuric acid.)

A. Blood.

1. (a) A whole blood filtrate is prepared according to the usual Somogyi procedure (17). Two ml. of filtrate (representing 0.2 ml. of whole blood) is placed in a 25-ml. Erlenmeyer flask to which is added 0.3 ml. of 1 M acetate buffer, pH 5.5, and 0.5 ml. of glucose oxidase solution, containing 2 mg. of enzyme per ml. of distilled water.
   (b) A second flask is prepared identically except that 0.5 ml. of water is added in place of the enzyme. This flask gives the total reducing value.
   (c) An enzyme blank is set up containing 2 ml. of distilled water, 0.3 ml. of buffer, and 0.5 ml. of enzyme solution.
2. All flasks are incubated under O_2 with agitation for three hours at 39° C., in a Dubnoff metabolic shaker.
3. The contents of all flasks are centrifuged when particulate enzymatic material is present. Each supernatant solution is analyzed, in duplicate, for reducing material according to the procedure of Nelson and Somogyi (18, 19), which required 0.5 ml. of solution per determination. Duplicate aliquots of the enzyme-treated samples are used for determination of pentose with orcinol-ferric chloride according to Hurlbert, Schmitz, Brumm, and Potter (24).
4. Reference standards for calculations of true glucose content are determined for each experiment by measuring the copper reducing values of external glucose standards prepared in three different concentrations. Pentose levels are determined with reference to internal standards prepared in each experiment by adding a known amount of pentose to control blood.

B. Urine.

1. The procedure for urine is the same as for blood except that urine is diluted 1:25, or more if necessary, to keep glucose content less than 1.5 mg. per ml.
2. Two ml. of appropriately diluted urine was used instead of blood filtrate as outlined in the method for the blood assay above.

Recoveries

Known amounts of glucose or of pentose were added to whole blood in quantities simulating concentrations encountered experimentally. Recoveries of glucose averaged 94 per cent; of D-xylose, 97 per cent; of L-arabinose, 105 per cent; of D-arabinose, 101 per cent. Comparable recoveries were obtained from urine.

Comments on the method

1. *Use of filtrates.* We have found it convenient to prepare the Somogyi filtrates of blood, and freeze the filtrates until analyzed. Glucose and pentose are stable for at least eight weeks in this form. Froesch and Renold (5) have incubated hemolysates directly with large quantities of enzyme. The activity of glucose oxidase is somewhat greater in such hemolysates than in filtrates (8 per cent per hour, expressed as rate of glucose oxidation). However, our procedure was standardized in such a way as to permit complete destruction of glucose in blood concentrations ranging up to 1500 mg. per cent in three hours of incubation.

2. *Glucose oxidase.* Three different preparations were evaluated. Those purchased from Sigma Chemical Company and from Bios Biochemicals were somewhat more active by weight than that obtained from Squibb and Company.[2] In equivalent quantities, in terms of ac-

[2] The authors are indebted to Dr. David Perlman, Squibb and Company, New Brunswick, New Jersey, for a generous gift of glucose oxidase.

tivity, the contributions and reducing material by these preparations were about equal by the copper reduction method. The enzyme blanks averaged about 0.048 O.D. unit at 540 mμ in the Somogyi-Nelson method for all three preparations used. However, in the orcinol pentose method, the enzyme blank was 0.200 O.D. unit at 660 mμ for the Bios and Sigma material but only 0.027 O.D. unit for the Squibb preparation.

3. *Non-glucose reducing material in control blood.* There was an average of 1.5 mg. per cent of non-glucose reducing material (expressed as glucose) in the filtrate after glucose oxidase treatment, with a range of −4 to +5 mg. per cent. Similarly, there was an apparent average pentose concentration of 4.3 mg. per cent in fasting control blood specimens (Range, 2.6 to 6.6 mg. per cent), but since it is known that orcinol reacts with glucuronic acid (25) and certain other substances, and that the normal fasting glucuronic acid content of plasma ranges from 2.5 to 4.5 mg. per cent (26), this orcinol value cannot be regarded as representing pentose. The orcinol value obtained on control blood filtrates was subtracted from each subsequent value.

Other methods

Lactate was determined on blood filtrates according to the Mitchell and Cournand modification (27) of the method of Barker and Summerson (28). Pyruvate was determined on blood filtrates with lactic dehydrogenase according to Segal, Blair, and Wyngaarden (29). Inorganic phosphorus was determined on serum according to Fiske and Subbarow (30).

Expired CO_2 was collected for timed intervals of 3 to 5 minutes in a Douglas bag through a one-way valve arrangement. Room air was inspired. CO_2 and O_2 content of expired air were measured in a Cambridge CO_2 analyzer and a Beckman Model E2 oxygen analyzer, respectively.[3] The volume of expired air was measured in a Tissot spirometer, after removal of CO_2 by bubbling through CO_2-free N NaOH. Carbonate was collected by precipitation with $BaCl_2$. The $BaCO_3$ was washed with water till the supernatant solution was no longer alkaline, and dried *in vacuo* at 110° F. overnight. $BaCO_3$ was then pulverized in a mortar, transferred to stainless steel planchets as an alcohol suspension, and counted at infinite thickness in a Robinson proportional gas-flow counter (31) having a background of 2 to 4 c.p.m., and an efficiency at infinite thinness of 53 per cent. The cumulative excretion of $C^{14}O_2$ was then determined according to Berlin, Tolbert, and Lawrence (32).

Urinary C^{14} excretion was determined by counting urine in a Tricarb Liquid Scintillation counter.[4] Two-tenths ml. of dilute urine, 2.8 ml. of alcohol, and 7.0 ml. of toluene containing 0.4 per cent 2,5-diphenyloxazole (33) were placed in a glass vial, mixed by gentle swirling, chilled, and counted to 1 per cent accuracy. Under these circumstances the overall efficiency of counting was 45 per cent.

Urine was chromatographed on Whatman No. 1 paper in a descending system for 24 hours employing water-saturated phenol (Mallinckrodt) as solvent. After overnight air drying, the papers were sprayed with bromcresol purple-boric acid-borax solution (34) for visualization of polyhydroxy compounds, and with aniline hydrogen phthalate in n-butanol (35) for visualization of aldoses. Compounds were eluted by descending chromatography with water; eluates were counted in the liquid scintillation counter as described above.

Periodate oxidation of urine containing C^{14}-xylose and possible metabolites was performed according to Eisenberg (36). Recrystallized dixanthydryl urea was prepared from urine according to Fosse (37).

RESULTS

Rates of disappearance from blood of pentoses administered intravenously

When blood levels[5] of D-xylose, D- or L-arabinose, and D-lyxose, determined at various time intervals following infusion, were plotted on semilogarithmic coordinates, straight lines were obtained. A typical experiment with each sugar is illustrated in Figure 1. These results indicate that the removal of these pentoses from the blood is a first order kinetic process. The deviation of the early concentration values from the logarithmic

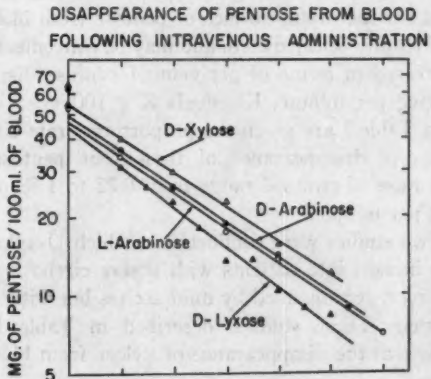

FIG. 1. DISAPPEARANCE OF PENTOSE FROM BLOOD FOLLOWING INTRAVENOUS ADMINISTRATION

In the studies shown 10 Gm. of pentose was infused, and blood levels are plotted against time on hemilogarithmic coordinates.

[3] The authors gratefully acknowledge the assistance of Mrs. Harriett Coburn and Dr. Ben Branscomb of the National Heart Institute for these determinations.

[4] Packard Instrument Company, LaGrange, Illinois.

[5] The orcinol reaction is not specific for pentoses, and metabolites of the infused pentoses may be included in these values.

TABLE I

Infusion of pentoses into normal subjects

Pentose infused	Quantity infused	Subject	Weight	Disappearance from blood	Volume of distribution	Urinary recovery	Blood glucose change
	Gm.		*Kg.*	*K' %/min.*	*liters*	*% of admin. dose*	*mg. %*
D-xylose	5	W. M.	59.0	1.58	16.6	44	+25
D-xylose	10	W. M.	59.0	0.89	18.7	49	+15
D-xylose	10	W. M.	59.0	1.28		46	+10
D-xylose	10	W. M.	59.0	1.33		34	+20
D-xylose	20	W. M.	59.0	0.89		38	0
D-xylose	10	W. M.	59.0	0.99	13.2	49	+ 3
D-xylose	10	W. R.	70.0	1.26	16.7	18	+32
D-xylose	10	W. R.	70.0	1.07	20.4	37	
D-xylose	20	W. R.	70.0	0.90	19.3	42	+25
D-xylose	20	W. R.	70.0	0.79	18.6	55	+30
D-xylose	10	J. B.	69.0	0.99	14.5	27	+21
D-xylose	10	A. R.	71.0	0.72	18.5	44	+20
D-xylose	10	A. R.	71.0	0.72	18.4	41	+12
D-arabinose	10	W. R.	70.0	1.06	15.1	36	+ 3
D-arabinose	10	S. B.	70.0	0.92	19.8	60	−10
D-arabinose	10	C. C.	60.0	0.85	11.0	56	+ 7
L-arabinose	10	W. R.	70.0	1.05	15.1	38	+11
L-arabinose	10	W. M.	59.0	0.77	13.9	38	+16
L-arabinose	10	S. B.	70.0	1.20	17.6	60	+18
L-arabinose	20	C. C.	60.0	0.94	20.0	52	+15
D-lyxose	10	S. S.	84.2	1.19	16.6	37	−10
D-lyxose	20	W. R.	70.0	1.10	15.3	55	−14

curve probably represents a temporary distribution gradient between blood and tissues. From a knowledge of the biological half-time, a rate constant for the disappearance of pentose from blood may readily be calculated, and may be conveniently expressed in terms of per cent of pentose disappearing per minute, K′ equals K × 100.

In Table I are given the proportional rate constants of disappearance of these four pentoses. The rates of removal range from 0.72 to 1.58 per cent per minute.

Two studies were conducted in which D-xylose was infused into patients with severe cirrhosis of the liver, accompanied by mild ascites but without icterus. These studies, described in Table II, show that the disappearance of xylose from blood

TABLE II

Rates of disappearance of infused xylose from blood in two patients with cirrhosis

Subject	Sex	Endogenous creatinine clearance	Quantity infused	Disappearance	Urinary recovery
		ml./min.	*Gm.*	*%/min.*	*% admin. dose*
McC.	M	92	10	0.43	47
D. R.	F	81	10	0.49	45

was considerably slower than normal in the two cirrhotic patients. Since the glomerular filtration rates (endogenous creatinine clearances) in these subjects were not greatly reduced, these results would suggest that the liver plays an important role in the removal of xylose from the blood.

Effect of pentose on blood levels of glucose, lactate, pyruvate, and inorganic phosphate

The fasting blood glucose values ranged from 43 to 96 mg. per 100 ml. in the normal subjects (Mean, 66 mg. per 100 ml). Following the infusion of 5 to 20 Gm. of xylose, the blood glucose values rose in at least 10 of 13 studies performed on four individuals. These rises ranged from 10 to 32 mg. per cent, but were not well correlated with the quantity of xylose infused. The maximum glucose values were consistently recorded 30 to 40 minutes after the end of the pentose infusions, and were followed by gradual declines of blood glucose values to or below the control concentrations. Examples of these rises of blood glucose may be seen in the companion paper by Segal, Wyngaarden, and Foley (13). Similar slight rises of blood glucose were encountered after in-

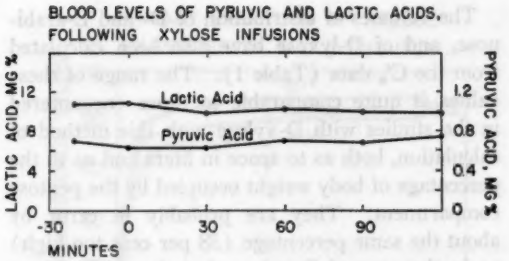

FIG. 2. BLOOD LEVELS OF PYRUVIC AND LACTIC ACIDS FOLLOWING INFUSIONS OF D-XYLOSE

The results plotted are averages of values obtained in six studies. The zero point on the time ordinate represents the end of the infusion.

fusions of L-arabinose (Table I). No significant increases of blood glucose were observed following infusions of D-arabinose or D-lyxose.

Blood lactate [6] and pyruvate concentrations [7] were not significantly or consistently altered by infusions of pentoses (Figure 2). Although only the results following xylose infusions are shown, they are also typical of those observed following infusions of the other pentoses. These results are in striking contrast to those obtained following infusions of glucose or of fructose, which regularly cause significant rises of both of these substances (38, 40).

The levels of inorganic phosphate in serum consistently declined during infusions of pentose. During the infusions of D-xylose, a mean fall of 0.42 mg. per cent (expressed as phosphorus) occurred. Following the infusion, the level of phosphate gradually rose and returned essentially to control levels in two hours. In patients receiving D- or L-arabinose, or D-lyxose, an initial fall of serum inorganic phosphate was also observed, but this fall was of lesser magnitude. The mean responses of serum inorganic phosphorus to infusions of 10 Gm. of D-xylose (five studies), and L-arabinose (three studies), 10 or 20 Gm. of D-arabinose (three studies), and 20 Gm. of D-lyxose (one study) are shown in Figure 3.

Distribution of xylose in body fluids

In two constant infusion experiments, the rates of urinary excretion of xylose were measured and compared with the rates of intravenous infusion. In the first experiment,[8] xylose was lost in the urine at a rate of 17.4 mg. per minute while being infused at a rate of 35.1 mg. per minute, over several periods when blood levels of xylose were nearly constant. In the second experiment, comparable values were 35.1 and 66.5 mg. per minute. Thus at equilibrium, 51 and 47 per cent, respectively, of the infused xylose were disappearing from the xylose compartment by non-renal mechanisms.

From knowledge of the composite rate of loss of xylose from its compartment at equilibrium (which rate is then equal to the rate of infusion), of the concentration of xylose in blood at equilibrium, and of the rate constant of xylose disappearance from blood (as determined following the completion of the xylose infusion), a volume of distri-

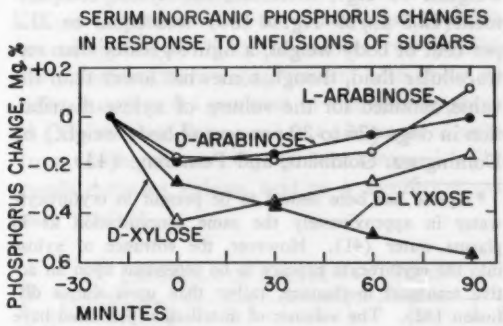

FIG. 3. AVERAGE SERUM INORGANIC PHOSPHORUS CHANGES IN RESPONSE TO INTRAVENOUS INFUSION OF PENTOSES

D-xylose and L-arabinose were given in 10-Gm. quantities; D-arabinose and D-lyxose in 20-Gm. quantities.

[6] Lactate and pyruvate values were determined following xylose infusion (including 2- to 20-Gm. infusion studies) in six instances, and following D-arabinose, L-arabinose and D-lyxose infusion in two instances each.

[7] The apparent constancy of the lactate/pyruvate ratio in blood has impressed earlier workers (38, 39). In the present study this ratio has ranged from 9.5 to 18.0 in over 120 determinations on 10 individuals, including control, cirrhotic and diabetic subjects. The mean value was 14.8, a value higher than that obtained by previous workers. This discrepancy reflects the lower values obtained for pyruvate with the specific enzymatic method than with the older procedures.

[8] In this study 5 Gm. of xylose was infused as a priming dose over a 20-minute period, followed by an additional 5 Gm. of xylose at a rate of 35.1 mg. per minute. In the second constant infusion study the quantity of primer and the rate of infusion were doubled.

bution [9] of the xylose present in the body during equilibrium may be calculated.

Let V = Volume of distribution at equilibrium, in L.
C′ = Concentration of xylose in plasma water at equilibrium, in mg. per L. = C/0.80.
K = Rate of disappearance of xylose from its compartment, in fraction per minute.
R = Rate of xylose infusion at equilibrium, in mg. per minute.

Then,

$$VC' = R$$
$$V = R/C'K$$

By this method,[10] volumes of distribution of xylose of 11.5 and 13.4 liters were calculated at equilibrium for the two constant infusion experiments. These volumes were smaller than those calculated from C'_0 values obtained by graphic extrapolation of the xylose disappearance curved to t_0, which averaged 17.3 liters [11] in 10 studies on four subjects (Table I). This discrepancy may be attributed to the inherent difficulty of knowing C_0 accurately by this method when the test substance has fates other than simple distribution and excretion (46, 47). The subject of this study weighed 59 Kg.; therefore, the xylose compartment (12.5 L./59 Kg. × 100) was equal to 21.2 per cent of body weight, a figure greater than extracellular fluid, though somewhat lower than the value obtained for the volume of xylose distribution in dogs (26 to 30 per cent of body weight) by Dominguez, Goldblatt, and Pomerene (43).

[9] Xylose has been shown to be present in erythrocyte water in approximately the same concentration as in plasma water (41). However, the entrance of xylose into the erythrocyte appears to be dependent upon an active transport mechanism, rather than upon simple diffusion (42). The volumes of distribution presented have been calculated on an assumption of equal distribution throughout intra- and extra-cellular water of blood. The concentration of xylose in plasma water (C′) has been calculated from the analytical value on whole blood (C) divided by 0.8, the approximate water content of blood.

[10] For a theoretical development of these intuited relationships see Dominguez, Goldblatt, and Pomerene (43), and Jokipii and Turpeinen (44).

[11] $V = Q/C'_0$, in which $C'_0 = C_0/0.8$, the concentration of pentose in plasma water (see text) at the theoretical moment of mixing, and Q is the quantity infused (45).

The volumes of distribution of D- and L-arabinose, and of D-lyxose have also been calculated from the C'_0 data (Table I). The range of these values is quite comparable to those encountered in the studies with D-xylose with this method of calculation, both as to space in liters and as to the percentage of body weight occupied by the pentose compartment. They are probably in error by about the same percentage (38 per cent too high) as in the case of D-xylose, but they do indicate that all of the pentoses studied are largely distributed in extracellular fluid. In this connection it is of interest to note that D-xylose has been shown to penetrate hepatic and renal tissue but not appreciably into muscle in the rat (48) under ordinary circumstances.

Urinary excretion of pentoses

The urinary excretion of pentoses was followed for 24 hours in almost all subjects. In control subjects known to excrete only trivial amounts of glucose normally, pentose was measured directly by the orcinol method without employment of glucose oxidase. In a few studies pentose excretion was checked by the more specific but far less sensitive *p*-bromaniline method (49), and excellent agreement with the orcinol results was found.

The urinary recoveries of infused pentoses are also shown in Table I. Approximately one-third to one-half of the quantity infused was recovered in urine as orcinol reactive material, and again there is no striking departure from this range for any specific pentose, although considerable variation is noted. The mean urinary excretion for all the pentoses was 43.5 per cent of the administered dose (Range, 27 to 60 per cent, neglecting one value of 18 per cent, which is probably in error). It is of interest that in the cirrhotic subjects (Table II) the urinary recoveries of infused xylose are well within the normal range.

Disposition of label following intravenous injection of xylose-1-C^{14}

In an effort to obtain information on the fate of the relatively large fraction of xylose which does not appear as orcinol reactive products in urine following its intravenous infusion, two experiments were performed in which the disposition of the isotope was determined following intravenous ad-

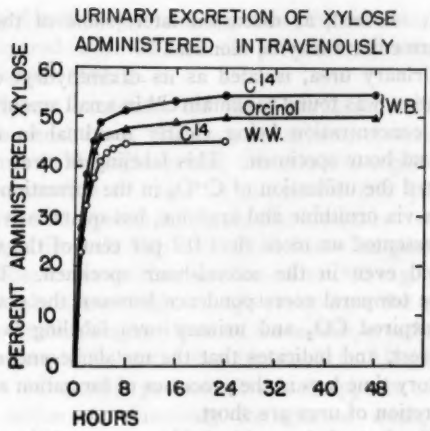

FIG. 4. CUMULATIVE URINARY EXCRETION OF PENTOSE AND OF C^{14} FOLLOWING INTRAVENOUS ADMINISTRATION OF D-XYLOSE-1-C^{14}

Subject W.B. received 20 Gm. of D-xylose containing 4.85 μc. of C^{14}. Subject W.W. received 5.7 mg. of D-xylose containing 3.8 μc. of C^{14}.

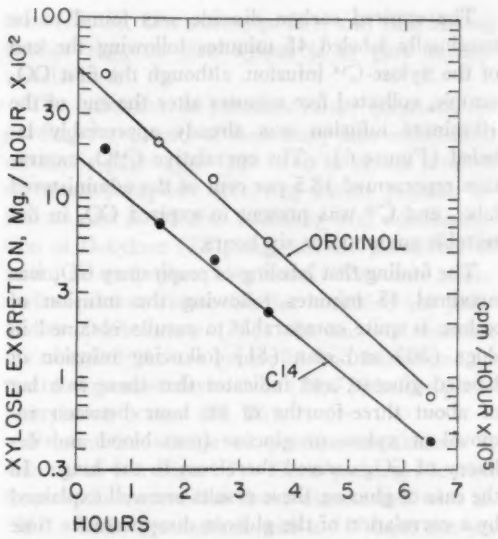

FIG. 5. RATES OF URINARY EXCRETION OF PENTOSE AND OF C^{14} FOLLOWING INTRAVENOUS ADMINISTRATION OF D-XYLOSE-1-C^{14}

This subject (W.B.) received 20 Gm. of D-xylose containing 4.85 μc. of D-xylose-1-C^{14} (5.7×10^6 c.p.m.).

ministration of xylose-1-C^{14}. In one study 4.85 μc. of xylose-1-C^{14} and 20 Gm. of unlabeled xylose were infused from the same flask, and the appearance of label as expired $C^{14}O_2$ was determined, as well as the excretion of label in hourly urine specimens. The concomitant administration of carrier xylose permitted comparison of urinary excretions of C^{14} and of orcinol reactive products in each specimen. In a second study, 3.8 μc. of xylose-1-C^{14} was infused as a tracer dose (5.7 mg.) into a second individual and only urinary excretion of label was determined. The results are shown in Figures 4, 5, and 6. Figure 4 shows that the cumulative urinary excretion of administered xylose was 54 per cent, measured as C^{14}, and 50 per cent, measured by orcinol reaction. The C^{14} value may include traces of xylose metabolites, including traces of labeled bicarbonate and urea. In the second study, 45 per cent of the label appeared in urine, so that both load and tracer doses of xylose appear to be handled similarly in the body. In Figure 5 are plotted the rates of urinary excretion of xylose, measured by C^{14} and orcinol methods. These results again indicate that the disappearance of xylose from blood is in part due to a first order excretion process. The half-time of xylose excretion in urine is of the same order as that of its disappearance from blood.

When urine was chromatographed, virtually all of the C^{14} recovered was found in the xylose band, suggesting that the orcinol reactive substance represented unaltered xylose, and less than 0.1 per cent traveled in the bands opposite markers of xylitol or D-xylonolactone, two possible early metabolites. In agreement with these results, less than 0.1 per cent of the label appeared in CO_2 obtained from periodate treatment of urine from which gaseous CO_2 had been removed. This result excludes xylonic acid as a significant urinary product.

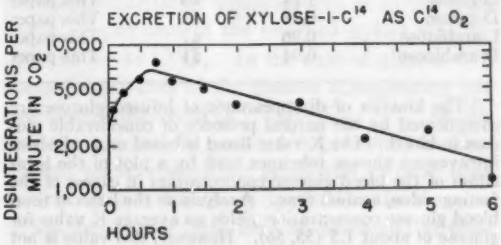

FIG. 6. EXCRETION OF D-XYLOSE-1-$C^{14}O_2$

The total C^{14} excreted in CO_2, expressed as disintegrations per minute in a one-minute sample of CO_2, has been plotted against time in hours.

The expired carbon dioxide was found to be maximally labeled 45 minutes following the end of the xylose-C^{14} infusion, although the first CO_2 sample, collected five minutes after the end of the 10-minute infusion was already appreciably labeled (Figure 6). The cumulative $C^{14}O_2$ expiration represented 13.5 per cent of the administered label, and C^{14} was present in expired CO_2 in detectable amounts for six hours.

The finding that labeling of respiratory CO_2 was maximal 45 minutes following the infusion of xylose is quite comparable to results obtained in dogs (50) and man (51) following infusion of labeled glucose, and indicates that there is a lag of about three-fourths of an hour between removal of xylose or glucose from blood and delivery of CO_2 derived therefrom to the lung. In the case of glucose, these results are well explained by a correlation of the glucose disappearance time and the half-time of the bicarbonate pool turnover which is 30 to 45 minutes in dogs (50, Discussion, p. 52) and man (52). The similarity of the CO_2 labeling pattern following infusion of labeled xylose probably indicates that the intermediary pools between xylose and CO_2 have rapid turnovers, as in the case of glucose (50, Discussion, p. 52). Indeed, as discussed later, some of these intermediates may be identical.

Urinary urea, isolated as its dixanthydryl derivative, was found to contain C^{14} in small amounts, the concentration being clearly maximal in the second-hour specimen. This labeling of urea reflected the utilization of $C^{14}O_2$ in the formation of urea via ornithine and arginine, but quantitatively represented no more than 0.2 per cent of the C^{14} found even in the second-hour specimen. The close temporal correspondence between the peaks of expired CO_2 and urinary urea labeling is of interest, and indicates that the metabolic and excretory time lags in the processes of formation and excretion of urea are short.

The fate of the residual 32 per cent of the administered label is obscure, as is that of the lower four carbons of the xylose whose first carbon appeared as CO_2. Attempts to follow C^{14} excretion in later urine specimens showed that no more than 0.2 per cent of the administered isotope appeared on days 2 or 3.

TABLE III

Comparison of rates of disappearances from pentoses and hexoses from blood

Sugar	Rate of disappearance from blood	Urinary excretion	Reference
	%/min.	*% of admin. dose*	
D-galactose	6.93	10	(52)
D-fructose	3.84	4	(38)
D-glucose	3.47*	2	(38, 53)
D-mannose	1.82	45	(54)
D-lyxose	1.14	46	This paper
D-xylose	0.98	42	This paper
L-arabinose	0.96	47	This paper
D-arabinose	0.94	51	This paper

* The kinetics of disappearance of infused glucose are complicated by the natural presence of considerable glucose in blood. The K value listed is based on analysis of intravenous glucose tolerance tests by a plot of the logarithm of the blood glucose concentration in excess of the fasting value, against time. Analysis on the basis of total blood glucose concentration yields an average K value for glucose of about 1.5 (55, 56). However, this value is not constant, but increases as the glucose load increases, whereas the K value calculated from the glucose increment data is constant at various glucose loads (56). We are in agreement with Duncan (56) that calculations employing glucose increments are based on a more reasonable physiological premise.

DISCUSSION

The rates of disappearance from blood of the pentoses D-xylose, D-arabinose, L-arabinose, and D-lyxose are considerably slower than those of the hexoses D-glucose, D-fructose, D-galactose, or D-mannose. Table III lists the half-times of disappearance of these sugars from blood following intravenous injection, in order of decreasing rate. It is of interest that there is a rather close correlation between the order of these rates and of the order of rates of absorption from the intestinal lumen. In order of decreasing rate of absorption, the data show D-galactose, D-glucose, D-fructose, D-xylose, D-mannose, L-xylose, L-arabinose (57–59). This apparent similarity need not, of course, imply common mechanisms.

In the present study the mean excretion of intravenously administered D-xylose was 42 per cent, of D-arabinose, 51 per cent, of L-arabinose, 47 per cent, and of D-lyxose, 46 per cent. These figures in man are in good agreement with the few published values of D-xylose and L-arabinose recoveries in man (41, 60), and with limited animal data obtained with D-xylose in dogs (43) and rabbits (61, 62), and with L-arabinose in rabbits (63). It is of interest also that intravenously administered L-xylulose is well utilized by the dog,

only 6 and 14 per cent appearing in the urine as compared with 60 and 65 per cent of infused D-xylose (64).

Xylose excretion has been carefully studied at a renal level. Almost no urinary excretion occurs in the aglomerular fish whereas rapid renal excretion is found in glomerular fish (65). In several species xylose/inulin clearance ratios ranged from 0.60 to 0.82 (66), but when plasma glucose concentration was raised to 300 mg. per 100 ml., a level high enough to saturate the glucose reabsorbing mechanism, the xylose/creatinine clearance ratio in the dog increased to 1.0 (67). The reabsorptive process, therefore, appears to be an active one involving the glucose transport system.

In the dog, xylose excretion has been found to be linearly related to plasma concentration (43). From our data also it would appear that the logarithmic disappearance curve of xylose from plasma is in part attributable to a first order excretion process. No work has been published relating to the renal handling of the arabinoses or of lyxose to the authors' knowledge.

The fate of the pentoses escaping rapid excretion required consideration. Early work in animals failed to show clearly that the pentoses were converted to glycogen. Although such a fate was claimed for D-xylose in the rat by Thomas, Gradinescu, and Imas (68), this was not confirmed by Miller and Lewis (69). And, although Rice and Roe (70) found D-arabinose administration to increase liver glycogen in fasted rabbits, Bloom and Stetten (71) found that D-arabinose-1-C^{14} and D-arabinose-5-C^{14} did not give rise to significant yields of $C^{14}O_2$ either when incubated with rat liver slices or when administered to intact rats and rabbits.. In our studies there do appear to be slight increases of blood glucose following infusions of D-xylose and L-arabinose. Similarly, Brien, Turner, Watson, and Geddes (72) report rises of blood glucose following 25-Gm. oral doses of D-xylose. Metabolism of xylose has now been demonstrated clearly. Since C^{14} appeared in CO_2 within 15 minutes of the start of the infusion of labeled xylose, it is not likely that bacterial degradation of xylose in the gastrointestinal canal can be held responsible. Furthermore, the kinetic characteristics of appearance of C^{14} in CO_2 are comparable to those found after glucose-C^{14} infusion (51, 50, Discussion, p. 52), and this finding further suggests that xylose is in part rapidly metabolized.

The utilization of pentoses raises questions regarding the manner of their entry into mammalian metabolic pathways. The drop in serum level of inorganic phosphate occurring during pentose infusions and noted also following oral administration of D-xylose (72), suggests the possibility of initial or early phosphorylation. The only mammalian pentose kinase thus far identified and purified is D-ribokinase (73). In bacterial systems the aldopentoses are generally first isomerized to their ketopentose derivatives (74, 75) before phosphorylation occurs (75, 76).

Present knowledge of pentose metabolism is summarized in Figure 7. Those pathways known to occur in mammalian tissue are marked with an "M." Pathways marked with a "?" have not yet been demonstrated to occur in biological systems but are suspected to exist. Unmarked pathways are those known to exist in plant or bacterial systems but not demonstrated in mammalian systems.[12] Free pentoses employed in this study are underscored for emphasis.

A reasonable hypothesis for the entry of D-xylose into general pathways of mammalian carbohydrate metabolism can be offered. D-xylose is converted to D-xylulose by xylose isomerase (75, 76). D-xylulose is then phosphorylated at the expense of ATP to form D-xylulose-5-phosphate (77). The number 1 carbon atom of xylulose phosphate (corresponding to the position of C^{14} in the xylose-C^{14} employed) eventually becomes C-1 of fructose-6-phosphate and would be lost as CO_2 in either the phosphogluconic acid oxidation cycle or in the Krebs cycle. During the period of logarithmic disappearance of xylose from blood, 27 per cent of the retained xylose was accounted for as $C^{14}O_2$. In the case of glucose-C^{14}, about 60 per cent of the glucose disappearing per unit time is delivered as C^{14} to CO_2 (51). In this case the residual 40 per cent is presumed to have entered non-carbohydrate pools and to have been utilized in part for tissue synthesis. In the case of xylose, similar fates may be presumed at least in part, since by known pathways a portion of the

[12] The authors are indebted to Dr. Bernard L. Horecker for helpful discussions in constructing and labeling this schematic diagram.

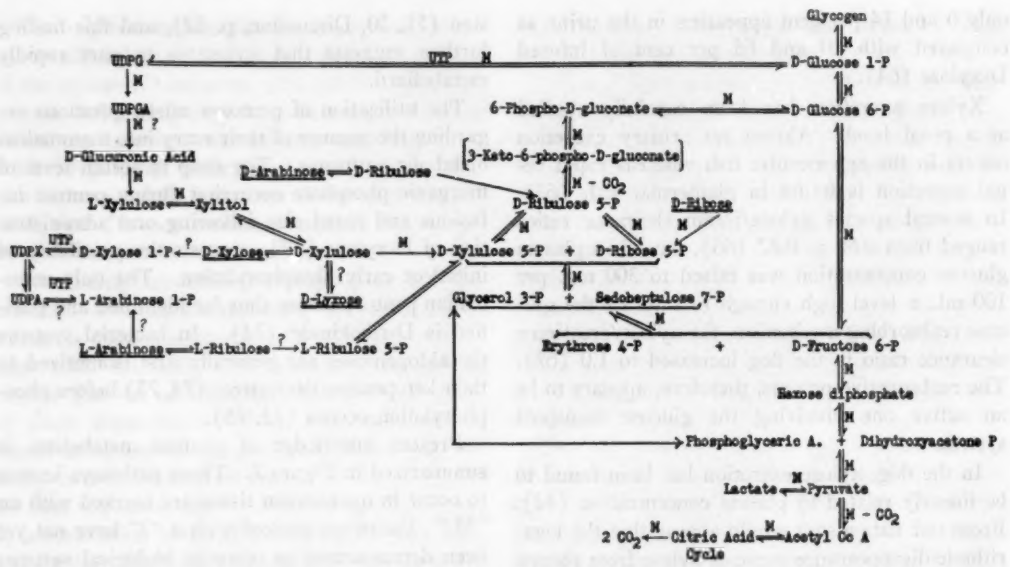

FIG. 7. SCHEMATIC DIAGRAM OF PATHWAYS OF PENTOSE METABOLISM

Pathways marked with an "M" have been demonstrated to occur in mammalian systems. Pathways marked with a "?" have not been demonstrated to occur in any biological system, yet are thought probable on the basis of related reactions. Pathways not marked with either symbol are known bacterial or plant reactions, but have not as yet been demonstrated in mammalian tissues. UTP = uridine triphosphate. UDPG = uridine diphosphate glucose. UDPGA, UDPX and UDPA = uridine diphosphate glucuronic acid, uridine diphosphate xylose and uridine diphosphate arabinose, respectively. The brackets around 3-keto-6-phospho-D-gluconate indicate that this intermediate has not yet been isolated.

metabolized xylose would yield glucose-6-phosphate as a product. The observations that serum inorganic phosphate falls during xylose infusion, that blood glucose tends to rise slightly 30 to 45 minutes later, and that no change of blood pyruvate or lactate occurs are all in keeping with the proposed interpretations of xylose utilization. The lack of lactate or pyruvate rises may be a reflection of the slowness of conversion of xylose to gluose, and is reminiscent of a similar lack of change of lactate or pyruvate following intravenous D-mannose infusions (54), even though the phosphorylation of mannose (78) and subsequent conversion to glucose or fructose esters are well known mammalian reactions (79).

In the case of the arabinoses also, the reactions upon which a tentative interpretation may be predicated (Figure 7) have not all been demonstrated to occur in mammalian tissue, yet they appear likely mechanisms to explain the metabolism of the non-excreted fractions of the infused sugars, and all are known biological reactions. The possibility of pentose transformations as components of nucleotides (80) must also be borne in mind.

Almost no information exists regarding pathways of metabolism of D-lyxose. A possible point of entry into the known metabolic channels is via D-xylulose. Isomerization of the pentoses D-xylose ⇌ D-xylulose ⇌ D-lyxose, suggested by this scheme, would be analogous to that occurring with D-glucose ⇌ D-fructose ⇌ D-mannose, the respective hexose analogues of the three pentoses. The hexose tautomerizations actually involve the phosphate esters rather than the free sugars (79), and tautomerization of the pentoses as phosphate esters is another possibility. By either of these proposed routes D-lyxose might be anticipated to have approximately the same metabolic fates as D-xylose.

In addition to these pentose pathways, there is scattered evidence for the existence of various mammalian pentose dehydrogenases. D-glucose dehydrogenase of liver acts on D-glucose, galactose, arabinose, and xylose (81, 82); a partially

purified dehydrogenase of lamb's liver acts on D-glucose, D-arabinose, D-lyxose, and possibly D-xylose, as well as on certain phosphorylated hexoses (83); a dehydrogenase from cat liver acts on D-glucose, D-arabinose, glyceraldehyde, glycolaldehyde, and possible D-erythrose (84). The products of the dehydrogenation of pentoses are unknown, but would probably involve pentonic acids, by analogy with the dehydrogenase reactions involving glucose (81). However, if xylonic acid is a product of such reactions in man, it must be further metabolized, for no evidence for its occurrence in urine was obtained in this study.

SUMMARY

Following their infusion intravenously into normal subjects, the aldopentoses D-xylose, D-arabinose, L-arabinose, and D-lyxose disappear from blood at rates proportional to concentration. The half-times of disappearance were similar for the four pentoses, and averaged 71 minutes. Approximately 40 per cent of the administered pentoses were recovered unchanged in the urine. The pentoses were shown to be distributed initially in a volume of body water somewhat larger than that of extracellular water. The rate of disappearance of xylose from blood was considerably slower than normal in two cirrhotic subjects.

The infusion of each of the pentoses was accompanied by a significant drop in the serum level of inorganic phosphate, but no change in the blood levels of lactate or pyruvate were observed. Slight rises of blood glucose levels were frequently observed following infusions of D-xylose or L-arabinose.

Limited studies with D-xylose-1-C^{14} have shown that this sugar is in part rapidly metabolized, giving rise to labeling of expired CO_2. No significant labeling of urinary products other than of the unchanged D-xylose was detected.

The metabolism of the non-excreted portions of these pentoses was discussed in terms of present concepts of carbohydrate metabolism in mammalian systems.

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STUDIES OF RIBOSE METABOLISM. I. THE PATHWAY OF NUCLEIC ACID RIBOSE SYNTHESIS IN A HUMAN CARCINOMA CELL IN TISSUE CULTURE [1]

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An impressive body of evidence is available demonstrating the importance of the pentose phosphate pathway (hexose monophosphate shunt) in the intermediary metabolism of carbohydrate (1). The pathway is involved in a number of vital biological processes, including photosynthesis (2), carbohydrate utilization by many bacteria (3), the elaboration of the ribose moiety of nucleic acids in the chick (4), in certain microorganisms (5–8), and in the rat (9, 10), the synthesis of the precursor of aromatic amino acids (11), and the generation of reduced triphosphopyridine nucleotides, which are required for the synthesis of several substances including fatty acids and cholesterol (12). In addition, it is the route by which a substantial fraction of glucose is catabolized by rat liver slices (13). Recent work has demonstrated its role in the *in vivo* conversion of ribose and xylose carbon to mouse liver glycogen (14).

The present investigation was undertaken to study the mechanism of nucleic acid ribose synthesis by a human carcinoma cell (strain HeLa) grown in tissue culture. The results are consistent with ribose production via both the oxidative and nonoxidative steps of the pentose phosphate pathway, with the nonoxidative route predominating.

METHODS

Tissue culture. A human carcinoma cell (strain HeLa) and a human liver cell (Henle, strain 407) were grown in the amino acid-vitamin-salt-glucose medium described by Eagle (15) supplemented with 5 per cent whole horse serum. After two or three days of incubation, at which time the inoculated cells were firmly adherent to the wall of the 1-liter Blake bottle in which they were grown, the C^{14}-labeled substrate was added in the amount described. The medium was renewed every second or third day, together with additional radioactive substrate. The period of growth varied from 7 to 14 days. The radioactive compounds in the amounts employed had no discernible effect on cellular morphology or on rate of growth. At the termination of the growth period the cells were scraped from the wall of the bottle with a rubber policeman into 5 ml. of cold 10 per cent trichloroacetic acid (TCA).

Isolation of ribose. The ribonucleotides were isolated by a modification (16) of the procedure of Schmidt and Thannhauser (17). The harvested HeLa cells were homogenized for two minutes in a Potter-Elvehjem teflon homogenizer in 10 per cent TCA at 0° C. After centrifugation the precipitate was washed with 5 ml. of cold 5 per cent TCA. The precipitate was then extracted consecutively with 8 ml. of cold ethanol, 8 ml. of ethanol at 25° C., and twice with 8 ml. of an ethanol: ether (3:1) mixture. The cellular residue with 5 mg. each of ribonucleic acid and of deoxyribonucleic acid as carrier was then extracted with 1 ml. of 10 per cent sodium chloride in a boiling water bath for 20 minutes. After centrifugation the residue was similarly extracted twice with 0.5 ml. of 10 per cent sodium chloride for 20 minutes. To the combined extracts two volumes of ethanol were added. This mixture was kept at 5° C. overnight. The white precipitate was centrifuged, washed with 1 ml. of cold ethanol and with 1 ml. of ether, and then dried in air. One ml. of 1 N KOH was added and the mixture incubated at 30° C. for 22 hours. Deoxyribonucleic acid was precipitated by acidification with sufficient H_2SO_4 to make a final concentration of 0.5 N, and removed by centrifugation. Dephosphorylation of the ribonucleotides, which remained in the supernatant solution, was carried out by adjusting the pH to 5.6, adding acetate buffer to make a final concentration of 0.15 M, and introducing 0.1 ml. of acid phosphatase (Worthington wheat germ phosphatase, 20 mg. per ml. of water). The mixture was incubated at 30° C. for two hours, and the reaction stopped by placing the tube in boiling water for two minutes. The pH of the solution was then adjusted to 6.0 with KOH. The nucleosides were hydrolyzed by incubation with 1 ml. of riboside hydrolase (18) at 34° C. for three hours. The reaction was stopped in boiling water, and the solution centrifuged. The supernatant solution was deionized by passage through a mixed bed resin (MB-3; Fisher Scientific Company). The effluent was lyophilized to dryness and the residue taken up in methanol. The methanol solution was streaked on Whatman No. 1 paper and the

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ribose purified by descending chromatography in N-butanol-glacial acetic acid-water (4:1:5) for 20 hours (19). A standard ribose solution was spotted on the paper lateral to both margins of the HeLa cell mixture. The position of the ribose was located by streaking with aniline phthalate reagent (20) the area of the paper over which the standard ribose solution had migrated and the adjacent margins of the incubation mixture. The strip containing the ribose was cut out and the sugar eluted with water.

Degradation of ribose. The RNA ribose, to which was added sufficient unlabeled ribose to make a total of 350 μM, was degraded to acetate (carbons 1 and 2 of ribose) and lactate (carbons 3, 4, and 5) by fermentation with *Lactobacillus pentosus* (21). The fermentation products were isolated as described by Bernstein (22). Acetate was converted to barium carbonate ($BaCO_3$) in stepwise fashion as described by Phares (23). Lactate was degraded to CO_2 (carbon 3 of ribose) and acetate (carbons 4 and 5) by the method of Aronoff, Haas, and Fries (24), and the acetate was subjected to the Phares procedure (23). The microbiological and chemical degradation techniques permitted the isolation of each carbon atom of the ribose molecule as $BaCO_3$. When ribose-1-C^{14} was degraded in this fashion, over 99 per cent of the total radioactivity was found in carbon 1.

Determination of radioactivity. Bicarbonate-C^{14}, the radioactive sugars, and the cellular extracts were counted at "infinite" thinness with a gas flow counter, operating at an efficiency of 40 per cent. $BaC^{14}O_3$ was counted at "infinite" thickness. All determinations were continued sufficiently long so as to reduce the counting error to less than 3 per cent.

Chemical and enzymatic procedures. Phosphate was determined by the method of Taussky and Shorr (25), and ribose by a modification (26) of the Mejbaum procedure (27). Riboside hydrolase was prepared from *Lactobacillus delbruckii*, according to the method of Takagi and Horecker (18). The activity of the preparation was assayed with inosine as substrate and xanthine oxidase, kindly provided by Dr. Boris Magasanik.

Materials. The initial stock cultures of HeLa cell and of a liver cell were generously provided by Dr. George Foley.

Bicarbonate-C^{14} was prepared by absorbing in NaOH $C^{14}O_2$ liberated from $BaC^{14}O_3$, and then neutralizing to the phenolphthalein end point. Glucose-1-C^{14} was purchased from Nuclear Chemical and Instrument Corporation, and glucose-2-C^{14} and ribose-1-C^{14} from the National Bureau of Standards. The glucose substrates were degraded as described previously (14) and the ribose-1-C^{14} by *Lactobacillus pentosus* fermentation in order to verify isotope distribution.

RESULTS

Incorporation of radioactive substrates

All of the C^{14}-substrates were incorporated into HeLa cell (Table I). Since the rate and amount of cell growth varied considerably from one study to another, quantitative conclusions cannot be drawn from the incorporation data. Radioactivity was distributed in several cellular extracts (Table I). When carrier glycogen was added to the cold TCA extract of HeLa cell grown in the presence of bicarbonate-C^{14} and the glycogen then recrystallized, no radioactivity was detected. In similar fashion, no glycogen formation could be detected in the human liver cell grown in tissue culture in the presence of $NaHC^{14}O_3$.

Isotope distribution in RNA ribose (Table II)

The ribose from cells grown in the presence of uniformly labeled glucose was also uniformly labeled. (This sample of ribose was isolated and kindly provided by Drs. B. L. Horecker and H. Eagle.) Ribose derived from glucose-1-C^{14} was predominantly labeled in carbon 1 with somewhat

TABLE I

Distribution of C^{14} in HeLa cell grown in presence of C^{14}-substrates

Experiment number	C^{14} substrate	Total μc. added	Incubation period (*days*)	Total c.p.m. in cell extracts		
				Cold TCA soluble	Alcohol and alcohol-ether soluble	Nucleotides
3	$NaHC^{14}O_3$	470	10	28,000	6,600	21,000
11	$NaHC^{14}O_3$	430	19	75,700	7,600	27,000
12	$NaHC^{14}O_3$	200	7	35,200	21,000	14,700
31	Glucose-2-C^{14}	14.8	6	18,900	68,000	27,000
38	Glucose-1-C^{14}	11	6	18,000	43,000	13,800
39	Glucose-1-C^{14}	22	10	12,200	52,000	59,200
41	Glucose-2-C^{14}	13	10	12,100	23,000	8,650
43	Glucose-2-C^{14}	13	6	15,800	38,000	21,900
44	Glucose-1-C^{14}	11	6	18,500	20,000	25,900
42	Ribose-1-C^{14}	10	4	10,400	19,500	39,700

TABLE II

C^{14} distribution in RNA ribose of HeLa cell grown in medium containing C^{14}-substrate

Experiment number	C^{14} substrate	Per cent total activity*				
		C-1	C-2	C-3	C-4	C-5
1	Glucose-U-C^{14}	19.9	20.1 (32.1)	20.0	20.0	20.0
39	Glucose-1-C^{14}	52.8 (6.55)	4.9	8.2	0.5	33.6
44	Glucose-1-C^{14}	47.8 (0.99)	4.2	8.0	2.8	37.2
31	Glucose-2-C^{14}	22.6	38.7 (1.76)	9.9	26.0	2.8
43	Glucose-2-C^{14}	23.3	37.0 (0.37)	11.6	22.3	5.8
42	Ribose-1-C^{14}	74.0 (5.80)	3.9	9.8	1.9	10.4

* The specific activity (in millimicrocuries per millimole of carbon) of the most radioactive carbon atom is given in parentheses.

less activity in carbon 5. The major sites of isotope localization in ribose synthesized from glucose-2-C^{14} were positions 2, 1, and 4, with the latter two carbon atoms less active than the former. Ribose from cells grown in the presence of ribose-1-C^{14} was most heavily labeled in carbon 1, but some randomization of isotope had taken place, particularly to carbons 3 and 5.

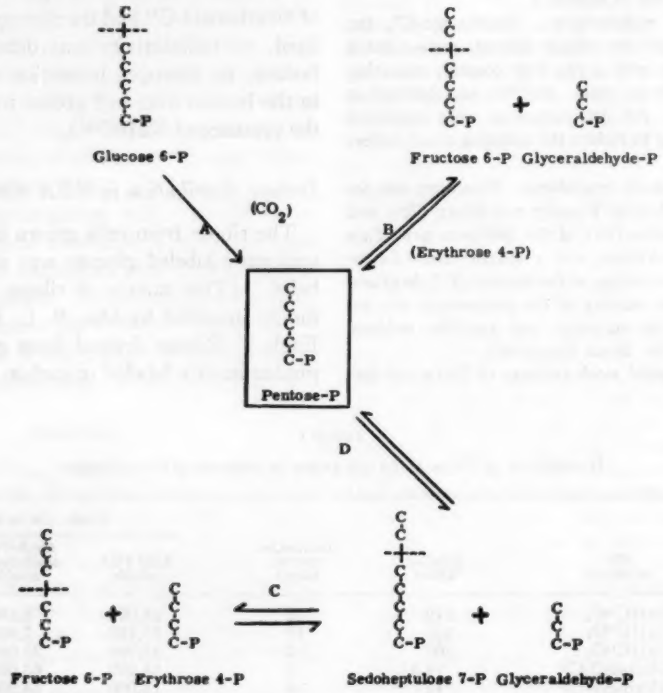


FIG. 1. PENTOSE PHOSPHATE SYNTHESIS VIA THE OXIDATIVE (A) AND NONOXIDATIVE STEPS (B, C, D) OF THE PENTOSE PHOSPHATE PATHWAY

The compounds in parentheses (steps A and B) are the reaction products other than pentose phosphate.

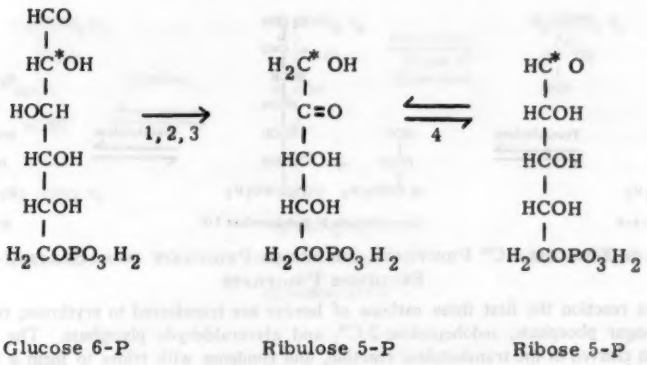


FIG. 2. PRODUCTION OF RIBOSE-1-C^{14} PHOSPHATE BY OXIDATIVE REMOVAL OF CARBON 1 OF GLUCOSE-2-C^{14} PHOSPHATE

C^{14} is indicated by the asterisk.

To exclude the possibility that the RNA ribose may have been contaminated by ribose-1-C^{14} substrate, 4 μc. of ribose-1-C^{14} were added to HeLa cells grown in the absence of C^{14} and treated with TCA. The nucleotide fraction isolated following the steps outlined above was found to be free of radioactivity.

DISCUSSION

Pentose phosphate may be generated by both oxidative and nonoxidative reactions of the pentose phosphate pathway (28) (Figure 1). In the oxidative steps carbon 1 of glucose 6-phosphate is converted to CO_2 and carbon atoms 2 to 5 to ribulose phosphate (Figures 1 (A) and 2). The nonoxidative reactions are described in Figures 1 (B, C, and D), 3, and 4. One nonoxidative reaction involves the transfer of the first two carbon atoms of a molecule of fructose 6-phosphate to an appropriate acceptor, such as glyceraldehyde phosphate (Figures 1(B) and 3) (29). This reaction is catalyzed by the enzyme, transketolase (30),

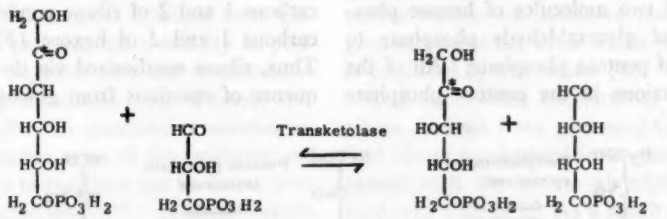


FIG. 3. PRODUCTION OF XYLULOSE-2-C^{14} PHOSPHATE FROM HEXOSE-2-C^{14} PHOSPHATE BY TRANSFER OF THE FIRST TWO CARBONS OF HEXOSE TO GLYCERALDEHYDE PHOSPHATE

The other product of this reaction, erythrose-4-phosphate, which is derived from carbons 3 to 6 of hexose, may then participate in the reactions shown in Figure 4.

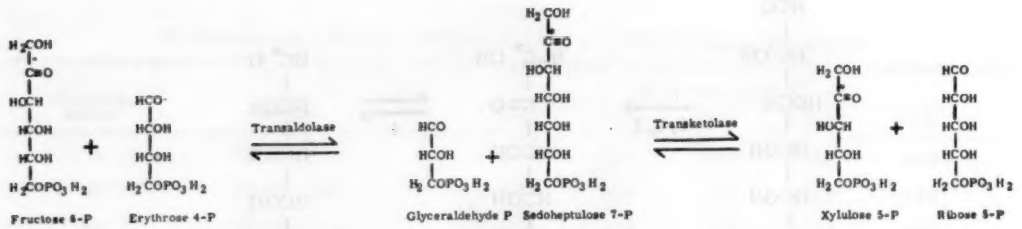


FIG. 4. PRODUCTION OF XYLULOSE-2-C^{14} PHOSPHATE AND RIBOSE PHOSPHATE FROM HEXOSE-2-C^{14} PHOSPHATE AND ERYTHROSE PHOSPHATE

In the transaldolase reaction the first three carbons of hexose are transferred to erythrose, resulting in the formation of a 7-carbon sugar phosphate, sedoheptulose-2-C^{14}, and glyceraldehyde phosphate. The first two carbons of sedoheptulose are then cleaved in the transketolase reaction, and condense with triose to form a molecule of xylulose-2-C^{14} phosphate, leaving a molecule of unlabeled ribose phosphate.

and the products are a molecule of pentose phosphate, xylulose 5-phosphate, and one of a 4-carbon sugar phosphate, erythrose 4-phosphate. The erythrose 4-phosphate may then act as acceptor for a 3-carbon unit cleaved from a second molecule of fructose 6-phosphate under the influence of transaldolase (Figures 1 (C) and 4) (31). The products of this reaction are a molecule of a 7-carbon sugar phosphate, sedoheptulose 7-phosphate, and one of triose phosphate. Transketolase may then mediate the transfer of the first two carbons of sedoheptulose phosphate to triose phosphate, yielding two molecules of pentose phosphate, one of xylulose 5-phosphate (32, 33) and one of ribose 5-phosphate (Figures 1 (D) and 4). To summarize these reactions (Figure 1), the oxidative steps in the pathway mediate the conversion of a molecule of hexose phosphate to one each of pentose phosphate and of CO_2. The transketolase-transaldolase reactions, on the other hand, catalyze the conversion of two molecules of hexose phosphate and one of glyceraldehyde phosphate to three molecules of pentose phosphate. All of the nonoxidative reactions in the pentose phosphate pathway are reversible, so that hexose monophosphate may be generated from pentose phosphate.

The enzymes responsible for the interconversion of the three pentose phosphates, xylulose, ribulose, and ribose, which arise in these reactions (Figure 5) are widespread in nature (34). Hence, both of the ketopentose esters, ribulose and xylulose, may be converted to ribose 5-phosphate. It is the latter which is involved in nucleotide synthesis (35).

Examination of the isotope distribution in HeLa cell ribose permits a recapitulation of the reactions by which the pentose was synthesized. If HeLa cell ribose were generated via oxidative loss of carbon 1 of hexose phosphate (Figure 2), the pentose derived from glucose-1-C^{14} would be unlabeled, and that obtained from glucose-2-C^{14} would be labeled in carbon 1. On the other hand, if the major pathway of ribose production involved the transketolase-transaldolase reactions, then carbons 1 and 2 of ribose would be derived from carbons 1 and 2 of hexose (Figures 3 and 4). Thus, ribose synthesized via the nonoxidative sequence of reactions from glucose-1-C^{14} would be

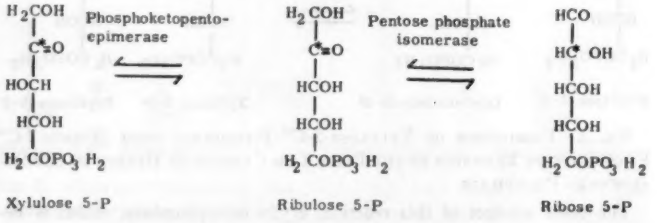


FIG. 5. INTERCONVERSION OF THE THREE PENTOSE PHOSPHATES INVOLVED IN THE PENTOSE PHOSPHATE PATHWAY

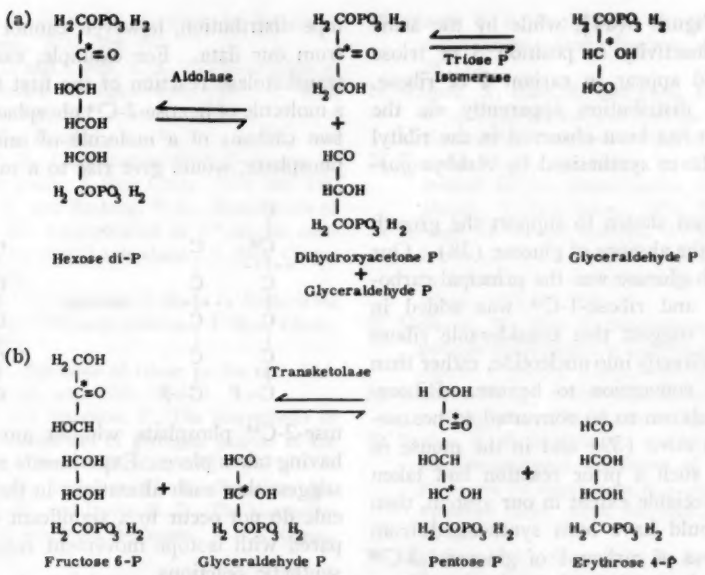


FIG. 6. (a) PRODUCTION OF GLYCERALDEHYDE-2-C^{14} PHOSPHATE FROM HEXOSE-2-C^{14} DIPHOSPHATE VIA THE ALDOLASE AND ISOMERASE REACTIONS (b) PRODUCTION OF PENTOSE-4-C^{14} PHOSPHATE FROM GLYCERALDEHYDE-2-C^{14} PHOSPHATE IN THE TRANSKETOLASE REACTION

Isotope in carbon 2 of the two-carbon donor in this reaction, *e.g.*, fructose-2-C^{14} phosphate, would, of course, appear in carbon 2 of the pentose phosphate.

labeled in position 1, and that obtained from glucose-2-C^{14} would have isotope in position 2. In the studies in which glucose-2-C^{14} was the HeLa cell substrate considerable radioactivity was found in *both* positions 1 and 2 of ribose. Therefore, both oxidative removal of the first carbon of hexose phosphate and the nonoxidative reactions may be presumed to have been operative in ribose production. The fact that ribose obtained from glucose-2-C^{14} had 50 per cent more activity in position 2 than in 1 suggests that the nonoxidative reactions are the predominant route of ribose synthesis in HeLa cell. No quantitative conclusions concerning the relative roles of the oxidative and nonoxidative series of reactions can be drawn from the studies with glucose-1-C^{14}. In these experiments only the participation of the transketolase reaction would be reflected in the isotope distribution in ribose, for any pentose synthesized via loss of carbon 1 of glucose-1-C^{14} would, of course, be unlabeled. Ribose synthesized via the transketolase reaction, however, would be labeled in carbon 1.

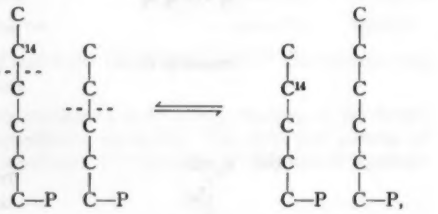
The participation of both oxidative and nonoxidative steps of the pentose phosphate pathway in ribose synthesis by HeLa cell is similar to reported mechanisms of nucleic acid ribose production *in vivo* by rat liver (9, 10) and of synthesis of the ribityl moiety of riboflavin in *Ashbya gossyppie* (7). Ribose synthesis by *Escherichia coli* (5, 8) and by *Torula utilis* (6), on the other hand, occurs mainly via oxidative loss of carbon 1 of glucose phosphate, while in the chick little pentose is produced by this means (4).

The substantial radioactivity in carbon 4 of ribose derived from glucose-2-C^{14} and in carbon 5 of ribose synthesized from glucose-1-C^{14} is consistent with the intense glycolytic activity of tumor cells (36, 37). As a result of aldolase cleavage of hexose diphosphate and isomerization of the triose phosphates, glucose-1-C^{14} and glucose-2-C^{14} would give rise to glyceraldehyde phosphate labeled in positions 3 and 2, respectively [Figure 6(a)]. The condensation of a two-carbon fragment with triose-2-C^{14} phosphate in the transketolase reaction would give rise to ribose with isotope

in carbon 4 [Figure 6(a)], while by the same mechanism radioactivity in position 3 of triose phosphate would appear in carbon 5 of ribose. Similar isotope distribution apparently via the aldolase reaction has been observed in the ribityl portion of riboflavin synthesized by *Ashbya gossyppie* (7).

Ribose has been shown to support the growth of HeLa cell in the absence of glucose (38). Our studies, in which glucose was the principal carbohydrate source and ribose-1-C^{14} was added in tracer quantity, suggest that considerable ribose is incorporated directly into nucleotide, rather than following prior conversion to hexose. Ribose-1-C^{14} has been shown to be converted to hexose-1,3-C^{14}, both *in vitro* (39) and in the mouse *in vivo* (14). If such a prior reaction had taken place to an appreciable extent in our system, then some ribose would have been synthesized from the oxidative loss of carbon 1 of glucose-1,3-C^{14} and would have been labeled in position 2. Further, aldolase cleavage of hexose-1,3-C^{14} would have given rise to triose-1,3-C^{14}. Condensation of a 2 carbon fragment in the transketolase reaction with triose-1,3-C^{14} would have resulted in radioactivity in positions 3 and 5 of ribose (Figure 6). The appearance of a relatively small concentration of isotope in those sites, particularly in positions 3 and 5 (Table II), suggests that some ribose-to-hexose conversion may have preceded ribose incorporation into nucleotide. The marked predominance of isotope in carbon 1, however, indicates that the major fraction of ribose-C^{14} was incorporated unchanged into nucleotide.

From our studies one cannot determine whether ribose is incorporated directly into nucleotide following its synthesis by HeLa cell. If incorporation is not direct, exchange reactions, as well as ribose to hexose to ribose recycling, could take place, and isotope distribution would thereby be altered. As a result, the pattern of radioactivity in the ribose molecule would reflect not only the reactions involved in net synthesis, but also those participating in exchange and recycling. From the experiment with ribose-1-C^{14} one may conclude that ribose to hexose to ribose recycling does not take place to a sufficient extent to alter markedly the isotope pattern in RNA ribose synthesized by HeLa cell under the conditions of our experiments. The effect of exchange reactions on isotope distribution, however, cannot be ascertained from our data. For example, exchange via the transketolase reaction of the first two carbons of a molecule of hexose-2-C^{14} phosphate with the first two carbons of a molecule of unlabeled pentose phosphate, would give rise to a molecule of pentose-2-C^{14} phosphate without any net synthesis having taken place. Experiments now in progress suggest that such alterations in the pentose molecule do not occur to a significant extent as compared with isotope movement resulting from net synthetic reactions.



SUMMARY

Nucleic acid ribose has been isolated from a human carcinoma cell (strain HeLa) grown in an amino acid-vitamin-salt-glucose medium containing C^{14}-labeled substrates. Isotope distribution in the ribose is consistent with its synthesis via both oxidative and nonoxidative steps of the pentose phosphate pathway. The nonoxidative mechanism appears to predominate. No glycogen synthesis was detected in HeLa cell or in a liver cell grown in tissue culture.

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PRODUCTION OF HYPERTONIC URINE IN THE ABSENCE OF PITUITARY ANTIDIURETIC HORMONE [1]

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The mechanism by which pituitary antidiuretic hormone (ADH) produces its renal effect has not been clearly established. Under ordinary conditions, in the absence of ADH, urine of a concentration considerably below that of plasma is produced in large volume. With increasing amounts of ADH, administered or secreted, the osmotic pressure of the urine rises to and above that of plasma. With respect to total water balance, the quantitatively more important contribution to water economy is the change between maximally dilute and isotonic urine, since this, in normal man, may involve a change in water excretion in excess of 10 ml. per minute. The further saving of water effected by the elaboration of hypertonic urine is a relatively small one, amounting, at usual rates of solute excretion, to not more than an additional 1 or 2 ml. per minute. Thus, although it is commonly stated that the function of ADH is to cause the excretion of a hypertonic urine, its more important function might be better defined as preventing the excretion of a dilute urine.

Recent studies of the effects of posterior pituitary extracts on frog skin have shown that these preparations markedly reduce the resistance of this membrane to the flow of water in response to gradients of osmotic pressure (1). Observations on frog bladder also suggest this mechanism of action (2). It would seem reasonable to examine the extent to which a similar mechanism of action could explain the effects of ADH on the concentration of the urine. Since the change from dilute to isotonic urine is clearly in the direction of the dissipation of an osmotic gradient, this effect of ADH could well be attributed to a change in the permeability of the tubule membrane to water. The change to hypertonicity cannot be so readily attributed to an effect on water permeability without some assumptions as to the mechanism by which the hypertonicity is produced. However, it is a reasonable hypothesis that the mechanism for the production of hypertonicity is not dependent upon ADH but that its effects are unmasked, in the presence of ADH, by 1) changes in the volume and concentration of urine delivered to it and, possibly, 2) changes in the permeability of the membranes separating the concentrating mechanism from the urine in the tubule lumen.

The most tenable view of the mechanism by which the urine is diluted is that the process involves abstraction of solute in a segment of the tubule, presumably the distal convoluted tubule, relatively impermeable to water (3); indeed, the results to be presented strongly support this assumption. The only solute available in quantities adequate to account for the volume of water freed of solute in the process of urine dilution is sodium and its equivalent anions. Anything which limits the amount of sodium salts reaching the diluting segment or the capacity of that segment to remove sodium and anion should reduce the capacity of the kidney to produce a dilute urine. Thus, in the absence of ADH, if one were able to reduce the volume of renal tubule fluid delivered to the diluting segment, it might be possible to limit the amount of water "freed" by the removal of the remaining sodium salts. Under these conditions the volume of fluid and particularly the volume of "free water" leaving the diluting segment and entering the final segment, believed to be the site of the production of hypertonicity, would be small. If the mechanism for rendering a urine hypertonic functions in the absence of ADH, it might be possible, then, to obtain urine hypertonic to plasma since removal of only a small amount of water would be required.

[1] The material in this paper was presented in summary form at the meeting of the American Society for Clinical Investigation, Atlantic City, May, 1956.

[2] Dr. Davidson's work was done under the tenure of an American Heart Association Fellowship.

The experimental investigation of this possibility requires reduction of the rate of glomerular filtration in the absence of antidiuretic hormone. The use of animals or patients with diabetes insipidus is subject to some uncertainty of interpretation since it is not possible ever to exclude with complete certainty some residual capacity to secrete ADH. Indeed, subject to this reservation, a number of observations made in diabetes insipidus have been wholly in accord with the premises set forth above (4–7).[3] It appeared probable that the most certain demonstration of the absence of ADH would be afforded by the presence of a normal kidney continuing to produce a urine of maximum dilution during the necessary manipulations upon the other kidney. This has been the design used in the experiments to be described.

MATERIAL AND METHODS

Experiments were performed on 12 female mongrel dogs, weighing approximately 20 kilos. The animals were prepared by a bladder-splitting procedure devised by Desautel (9). In this operation the bladder is separated from the urethra and divided longitudinally into two halves, each containing one ureteral orifice. Each half is then formed into a pouch and connected to the outside through a nylon funnel protruding through the skin of the abdomen through separate wounds. Urine from each kidney thus drains separately and continuously to the outside. The length of the nylon tube protruding through the abdominal wall was sufficient to prevent drainage directly onto the skin and to permit the connection of a short length of rubber catheter during urine collections. Between experiments the dogs were kept in metabolism cages so that the continuous drainage of urine left them relatively dry.

At the same time as the bladder-splitting operation, the right renal artery was approached through a separate incision in the flank. The artery was freed and any branches other than those to the kidney were ligated and severed. A small inflatable cuff of silicone rubber (silastic, Dow Corning) enclosed in a tantalum clip (Figure 1) was placed around the right renal artery and, in order to prevent extrusion of the artery upon inflation of the cuff, the ends of the tantalum clip were brought together with a loop of stainless steel wire. The silicone rubber cuff was then connected permanently to the exterior of the animal by a length of polyethylene tubing

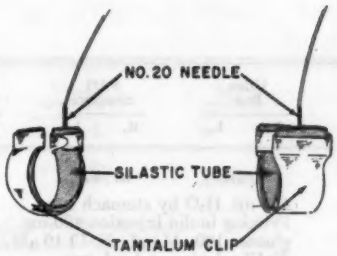

FIG. 1. INFLATABLE CUFF USED FOR COMPRESSION OF THE RIGHT RENAL ARTERY

(O.D., 1.27 mm.) brought out through a separate stab wound in the flank.[4]

Either before or after the surgical procedure the dogs were trained to stand quietly, partially supported by a sling under the chest and around each hind quarter. They were also trained to accept a stomach tube and venipuncture without interruption of a water diuresis. All experiments were performed on such trained animals without anesthesia.

Water diuresis was induced by the administration by stomach tube of approximately 30 ml. of water per kilo of body weight. The water load was then maintained by the continuous intravenous administration of an infusion at a rate estimated to approximate the expected urine flow and containing in addition to creatinine and/or inulin, *p*-aminohippurate (PAH) in amounts expected to insure complete extraction, mannitol at a concentration of 1 to 2 per cent in many of the experiments, small amounts of NaCl to replace anticipated losses in most of the studies, and sufficient glucose or fructose to bring the concentration of the solution to a non-hemolytic level.

Equality of renal function on the two sides was established by the collection of urine from each kidney during several periods before inflation of the renal artery cuff. In satisfactory surgical preparations, the filtration rates of the two kidneys were within 10 per cent of each other and in most instances within 5 per cent. The urine flow and concentration and the rates of excretion of sodium, potassium and chloride from the two kidneys were also very close to equal.

After collection of the control periods, the polyethylene tube communicating with the cuff was connected to a mercury manometer and the cuff inflated with air until the urine flow from the right kidney had been reduced to the desired level. This frequently required repeated adjustment of the pressure level since there was almost uniformly a greater depression of the urine flow during the first few minutes of compression than was apparent subsequently (10). When the desired level of urine flow had been obtained and maintained long enough to indicate

[3] In addition, since completion of these studies del Greco and de Wardener (8) have reported the production of hypertonic urine upon reduction of glomerular filtration rate under conditions in which the action of ADH was believed to be excluded.

[4] We are greatly indebted to Dr. Robert L. Bowman for devising and constructing the inflatable cuff, and to Drs. Jerome Kay and Edward Sharp for performing the surgical procedures.

TABLE I

Dog L, 3–29–56

Time	Urine flow R.	Urine flow L.	PAH clearance R.	PAH clearance L.	Inulin clearance R.	Inulin clearance L.	Urine osmolality R.	Urine osmolality L.	Solute excretion R.	Solute excretion L.	Plasma osmolality	Chloride excretion R.	Chloride excretion L.
min.	*ml./min.*		*ml./min.*		*ml./min.*		*mOsm./Kg. H_2O*		*μOsm./min.*		*mOsm./Kg. H_2O*	*μEq./min.*	
−57	600 ml. H_2O by stomach tube.												
−54	Priming inulin injection 400 mg. Start infusions a) inulin 1.5 mg./ml., mannitol 60 μM/ml., glucose 100 μM/ml., NaCl 10 μM/ml. in H_2O at 6.7 ml./min.; and b) PAH 5 mg./ml. in 0.4% NaCl solution at 1 ml./min.												
0–7	3.2	3.1	243	246	45	42	96	113	308	346	280	9	12
8–15	3.9	3.4	251	259	44	42	93	99	358	339		13	12
16–23	3.6	3.1	236	228	41	38	89	95	318	292	278	15	15
24–30	3.8	3.2	257	248	44	42	85	96	326	304		14	12
32	Cuff on right renal artery inflated.												
33	Mannitol in infusion a) replaced with equivalent amount of glucose.												
114–122	1.3	2.9	118	131	34	39	153	83	204	244	272	3	7
124–130	1.7	2.8	120	133	37	43	128	89	216	245	265	4	6
132	Cuff pressure readjusted.												
181–208	0.07	2.4	45	107	12	40	388	83	25	199	267	1	7
210–240	0.09	2.7	49	112	15	42	358	74	31	198	263	1	8
241–270	0.11	2.8	70	110	23	44	333	68	38	190	257	1	7
271–301	0.11	3.0	81	120	24	42	325	64	36	184	257	1	8
302–327	0.13	2.8	85	120	25	44	292	69	37	191	257	1.5	7

stability, sufficient time was allowed, before collection of urine, to minimize dead space errors due to changes in urine flow and concentration. (During these manipulations, urine flow from the control kidney remained unaffected.) After collection of several periods at a given level of pressure, the pressure was generally adjusted to another level and several additional periods collected. In other experiments, as indicated in the results, the content of the infusion was changed or vasopressin was administered by continuous infusion in a solution brought to pH 5.2 to 5.5 with acetic acid. In a few experiments, observations were made after deflation of the cuff; in these there was a prompt return of urine flow and concentration to control levels. Midpoint blood samples were

TABLE II

Dog S, 8–5–55

Time	Urine flow R.	Urine flow L.	PAH clearance R.	PAH clearance L.	Creatinine clearance R.	Creatinine clearance L.	Urine osmolality R.	Urine osmolality L.	Solute excretion R.	Solute excretion L.	Plasma osmolality	Chloride excretion R.	Chloride excretion L.
min.	*ml./min.*		*ml./min.*		*ml./min.*		*mOsm./Kg. H_2O*		*μOsm./min.*		*mOsm./Kg. H_2O*	*μEq./min.*	
−91	500 ml. H_2O by stomach tube.												
−62	Priming injection creatinine 2 Gm., PAH 20 mg. Start infusion creatinine 1 mg./ml., PAH 0.15 mg./ml., NaCl 20 μM/ml., mannitol 120 μM/ml. in H_2O at 11.7 ml./min.												
0–11	5.2	5.0	145	153	39	40	108	110	566	550		6.1	5.9
12–22	5.5	5.7	138	157	37	39	100	103	550	588	282	8.7	5.7
24	Cuff on right renal artery inflated.												
44–57	0.54	7.3	21	164	8	38	218	103	118	755	280	0.3	40
58–71	0.54	7.8	77	165	8	40	240	109	131	850	276	0.6	48
72	Pressure in cuff readjusted.												
82–90	2.3	8.8	112	188	18	41	150	114	348	997	278	6	69
91–98	2.6	8.8	181	181	22	39	168	115	442	1,006	276	6	65
99	Cuff deflated.												
107–112	6.1	7.5	146	134	33	35	117	118	714	878	276		32
143–147	5.9	6.3	102	103	31	32	124	122	728	774	282	14	13

TABLE III

Dog M, 1–16–56

Time	Urine flow R.	Urine flow L.	PAH clearance R.	PAH clearance L.	Inulin clearance R.	Inulin clearance L.	Urine osmolality R.	Urine osmolality L.	Solute excretion R.	Solute excretion L.	Plasma osmolality
min.	*ml./min.*		*ml./min.*		*ml./min.*		*mOsm./Kg. H_2O*		*μOsm./min.*		*mOsm./Kg. H_2O*
−69	750 ml. H_2O by stomach tube.										
−64	Start infusion: creatinine 2 mg./ml., inulin 2 mg./ml., PAH 0.7 mg./ml., NaCl 10 μM/ml., mannitol approx. 100 μM/ml., glucose approx. 85 μM/ml. at 6.9 ml./min.										
0–7	4.7	4.6	177	200	45	44	98	107	460	502	
8–15	5.1	5.2	195	208	46	47	98	102	504	531	283
16–23	4.8	4.9	176	192	43	45	98	106	470	515	
25	Replace mannitol in infusion with equivalent amount of glucose.										
27	Inflate cuff on right renal artery.										
56–62	1.5	4.9	164	164	38	47	212	106	326	523	280
65–72	1.1	4.8	145	160	33	47	235	102	258	492	279
76–83	0.96	4.4	118	150	30	45	240	96	230	420	277
83–88	1.6	4.2	114	147	31	48	162	98	252	412	277
89	Cuff pressure readjusted.										
102–110	0.85	4.0	115	152	33	48	251	98	214	388	276
120–132	0.66	4.0	112	130	33	47	285	93	189	369	275
138–148	0.58	4.0	95	115	31	46	302	87	175	348	274
151–162	0.54	4.0	86	112	27	46	271	81	145	324	273

collected from an indwelling venous needle in all periods.

Inulin was determined by the procedure of Walser, Davidson, and Orloff (11), creatinine by that of Kennedy, Hilton, and Berliner (12), *p*-aminohippurate (PAH) by the method of Bratton and Marshall (13), sodium and potassium by internal standard flame photometry, and chloride by electrometric titration. Urine and plasma osmolality was determined by the freezing point method using the apparatus described by Bowman, Trantham, and Caulfield (14).

RESULTS

Experiments involving inflation of the renal artery cuff during maximum water diuresis were performed in 12 dogs. The results were qualitatively the same in all. Every measurable depression of the rate of glomerular filtration was accompanied by a rise in the concentration of the urine from the experimental kidney. At some time in each of these dogs urine hypertonic to the plasma was obtained from the experimental kidney when the urine from the control kidney was indicative of maximum water diuresis. Illustrative experiments are represented in Tables I to III and the massed data from four of the dogs are plotted in Figures 2 to 5. In the figures, the ratio of urine osmolalities, experimental kidney to control kidney, is plotted against the corresponding ratio of glomerular filtration rates.[5] Points grouped about the ratio of 1.0 for both variables represent control periods before inflation of the cuff and indicate that there was no consistent difference between the two kidneys with respect to either rate of glomerular filtration or urine osmolality.

The data presented in Table I illustrate the

[5] In Dog S, only creatinine clearances were measured; in the other three dogs presented here, as well as in experiments upon two additional dogs, both creatinine and inulin clearances were measured. During control periods before application of pressure to the right renal artery the clearance of creatinine averaged 90 per cent of the inulin clearance for *both* kidneys. During inflation of the cuff the creatinine-inulin clearance ratio averaged 0.89 for the left (control) and 0.88 for the right (experimental) kidney. These creatinine-inulin clearance ratios are slightly lower than has been found previously (12), but, except for two aberrant periods (among approximately 100) yielding ratios of 0.58 and 0.61, there was no tendency for the clearances to diverge during the experimental manipulations. It is therefore concluded that changes in the clearance of either substance reflected similar changes in the rate of glomerular filtration. These results differ from those reported by Ladd, Liddle, and Gagnon (15), who found that the creatinine-inulin clearance ratio fell when one renal artery was clamped. However, the experimental conditions also differed in that the latters' experiments were done with hydropenic animals under anesthesia and subjected to surgical manipulations.

production of a rather marked degree of urinary concentration and maintenance of the concentration of the urine above that of plasma for a relatively prolonged period. In this experiment, the infusion of mannitol was discontinued at the time of inflation of the cuff and the rate of solute excretion was quite low during the period in which hypertonic urine was formed. At the time that the urine of highest osmolality was obtained the inulin clearance of the experimental kidney was some 70 per cent lower than that of the control kidney. The urine remained hypertonic to plasma over a period of two and a half hours, although the inulin clearance rose to approximately 55 per cent of control values.

Although urines of osmolality greater than 300 mOsm. per Kg. H_2O were regularly observed in these experiments, it was unusual to obtain urines with concentrations in excess of 350 mOsm. per Kg. H_2O and concentrations over 400 mOsm. per Kg. H_2O (up to 465) were observed in only two experiments in which the concentration of urine from the control kidney made it possible to exclude spontaneous secretion of antidiuretic hormone. Unfortunately, neither dog represented a satisfactory experimental preparation since one had chronic constriction of the right renal artery by the mechanical clamp used in some of the earliest experiments while the right kidney of the other dog functioned during control periods at about two-thirds the level of the normal kidney. The difference was subsequently shown at autopsy to be due to an infarct involving approximately one-third of the kidney. It is of interest that in this dog, although all the excretion rates were reduced by approximately one-third, the concentration of all the measured urinary constituents and the total osmolality were virtually identical to those of the normal kidney.

In the massed data it is apparent that there is marked variability 1) in the extent of the increase in osmolality obtained with a given reduction in glomerular filtration rate, and 2) in the extent of the reduction in glomerular filtration rate required to produce hypertonicity of the urine. Aside from a relatively small contribution of unavoidable dead space errors,[6] this variability is attributable largely

[6] A rise in the rate of urine formation during a collection period will make the urine appear more concentrated than that actually being formed and give a falsely high value for the inulin clearance. Decreases in urine flow have the opposite effect.

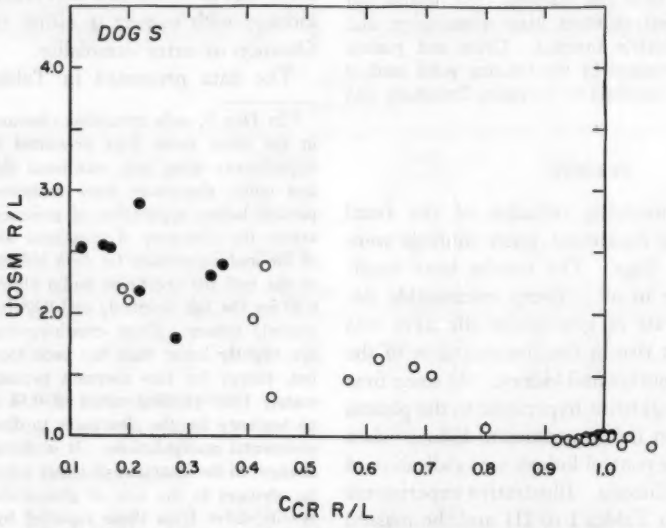

FIG. 2. RATIO OF URINE OSMOLALITIES (EXPERIMENTAL TO CONTROL KIDNEY) PLOTTED AGAINST CORRESPONDING RATIO OF EXOGENOUS CREATININE CLEARANCES

Filled circles indicate that the urine from the experimental kidney was hypertonic to plasma.

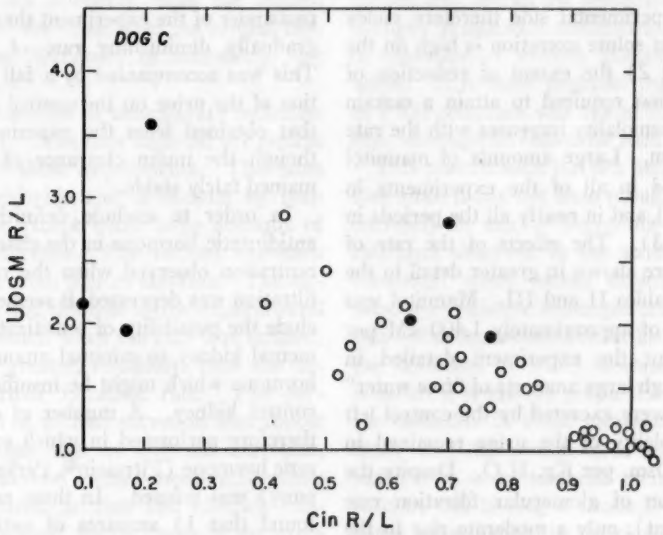

FIG. 3.

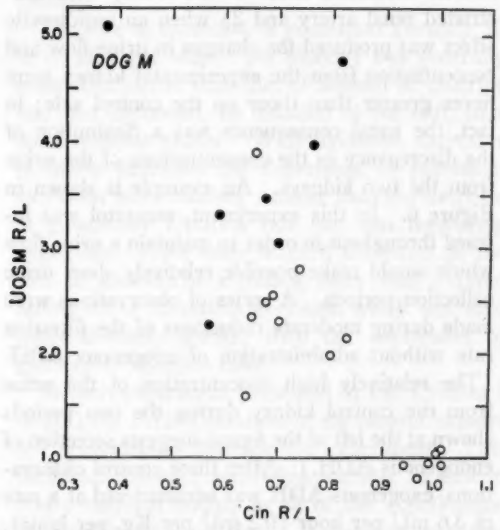

FIG. 4.

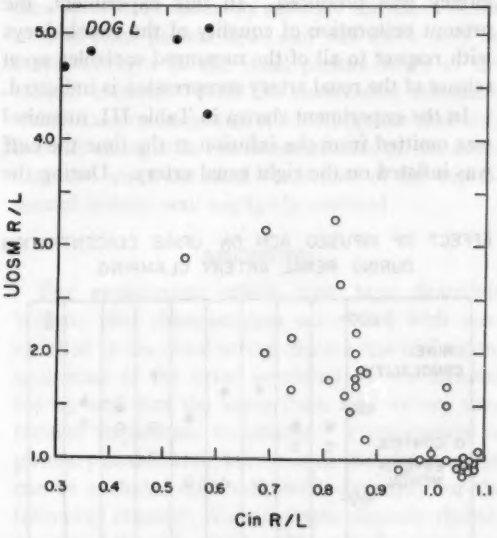

FIG. 5.

FIGS. 3–5. RATIO OF URINE OSMOLALITIES (EXPERIMENTAL TO CONTROL KIDNEY) PLOTTED AGAINST CORRESPONDING RATIO OF INULIN CLEARANCES

Filled circles indicate that the urine from the experimental kidney was hypertonic to plasma.

to variation in the rate of solute excretion; differences in solute output among different experiments and among experimental periods in the same experiment resulted in large measure from the rate at which mannitol was administered. The effect of solute excretion is exerted in two ways: 1) At high rates of solute excretion the urine from the control kidney, although containing large amounts of "free water," is less dilute than when the rate of solute excretion is lower; a given urine concen-

tration on the experimental side therefore yields a lower ratio when solute excretion is high on the control side; and 2) the extent of reduction of glomerular filtration required to attain a certain absolute level of osmolality increases with the rate of solute excretion. Large amounts of mannitol were administered in all of the experiments in Dog S (Figure 2) and in nearly all the periods in Dog C (Figure 3). The effects of the rate of solute excretion are shown in greater detail in the experiments in Tables II and III. Mannitol was infused at a rate of approximately 1,400 μM per minute throughout the experiment detailed in Table II. Although large amounts of "free water" (3.6 to 5.1 ml.) were excreted by the control left kidney, the osmolality of the urine remained in excess of 100 mOsm. per Kg. H_2O. Despite the marked depression of glomerular filtration rate (up to 80 per cent), only a moderate rise in the concentration of the urine from the experimental kidney was produced. In this experiment, the prompt restoration of equality of the two kidneys with respect to all of the measured variables upon release of the renal artery compression is indicated.

In the experiment shown in Table III, mannitol was omitted from the infusion at the time the cuff was inflated on the right renal artery. During the remainder of the experiment there was therefore a gradually diminishing rate of solute excretion. This was accompanied by a fall in the concentration of the urine on the control side and a rise in that obtained from the experimental kidney, although the inulin clearance of each kidney remained fairly stable.

In order to exclude definitively any role of antidiuretic hormone in the changes in urine concentration observed when the rate of glomerular filtration was depressed, it seemed desirable to exclude the possibility of sensitization of the experimental kidney to minimal amounts of circulating hormone which might be insufficient to affect the control kidney. A number of experiments were therefore performed in which exogenous antidiuretic hormone (Pitressin®, Parke, Davis and Company) was infused. In these experiments it was found that 1) amounts of antidiuretic hormone which produced no effect on the control kidney had no detectable effect on the kidney with constricted renal artery and 2) when an antidiuretic effect was produced the changes in urine flow and concentration from the experimental kidney were never greater than those on the control side; in fact, the usual consequence was a diminution of the discrepancy in the concentrations of the urine from the two kidneys. An example is shown in Figure 6. In this experiment, mannitol was infused throughout in order to maintain a urine flow which would make possible relatively short urine collection periods. A series of observations were made during moderate reductions of the filtration rate without administration of exogenous ADH. (The relatively high concentration of the urine from the control kidney during the two periods shown at the left of the figure suggests secretion of endogenous ADH.) After these control observations, exogenous ADH was administered at a rate of 3.6 mU per hour (0.2 mU per Kg. per hour), and after allowing time for achievement of a steady state, several additional periods were collected. The infusion rate was then doubled and the procedure repeated. In the latter group of periods only one was obtained in which the reduction of inulin clearance was comparable to that of the control periods. It may be seen that, although the concentration of the urine from the experimental kidney increased when Pitressin® was administered, the increment of osmolality was smaller,

EFFECT OF INFUSED ADH ON URINE CONCENTRATION DURING RENAL ARTERY CLAMPING

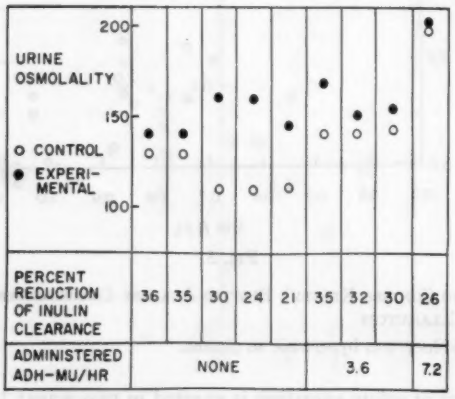

FIG. 6. OSMOLALITY OF URINE FROM CONTROL (OPEN CIRCLES) AND EXPERIMENTAL (FILLED CIRCLES) KIDNEYS DURING INFUSION OF VASOPRESSIN AT INDICATED RATES

Extent of reduction of inulin clearance by the experimental kidney is indicated for each period.

both absolutely and relatively, than the change in concentration of the urine from the control kidney and that the difference in osmolality was sharply diminished.

The rates of excretion of chloride are indicated in Tables I and II.[7] The data included are typical of the findings in these experiments. The rates of excretion of chloride and of sodium by both kidneys were low except when large amounts of mannitol were excreted. The sodium excretion was always lower than that of chloride. The excretion of sodium and chloride by the experimental kidney was depressed, when the cuff was inflated, to an extent far greater, relatively, than the decrease in glomerular filtration rate. The percentage reduction of sodium excretion was almost always greater than that of chloride excretion. Despite the rises in total solute concentration when the cuff was inflated, sodium and chloride concentrations were almost always lower in the urine from the experimental than from the control kidneys.

The rate of excretion of potassium (usually 10 to 20 μEq. per minute per kidney) was generally somewhat higher than that of sodium or chloride and was depressed, by inflation of the cuff, to a much smaller extent. With moderate decreases in inulin clearance the depression of potassium excretion was relatively smaller than the decrease in glomerular filtration rate and was frequently negligible. With more marked decreases in the rate of glomerular filtration there was a rapid falling off of potassium excretion which was then proportionately greater than the depression of glomerular filtration rate.

In each experiment presented in full (Tables I to III) the PAH clearances of the control kidneys fell progressively through the experiment. This decrease, although somewhat variable in extent, was a uniform finding in all of the experiments. Its significance is not clear. It seems unlikely that it represented an actual fall in renal plasma flow since decreases, often in excess of 50 per cent, were associated with no other systematic changes in the function of the control kidney which maintained a relatively constant rate of glomerular filtration and, within the limitations imposed by the changes in mannitol administration, constant rates of excretion of sodium, potassium, chloride and water. It is more probable that the gradual fall in PAH clearance was due to fall in PAH extraction, although no reason for such a fall is apparent.[8]

This phenomenon has not been investigated further since it has not been possible, with the PAH currently in use, to reproduce the fall in PAH clearance observed in the studies reported here. However, more recent experiments using the same manipulative technique confirm the observation of the experiments described in this paper that the reduction in inulin clearance, when the cuff is inflated, is always proportionately greater than the reduction in PAH clearance. It seems most likely that the administered PAH was contaminated with some material giving the same color reaction but having a much lower clearance and hence accumulating in the plasma in the course of the experiment. We believe that the ratio of PAH clearances is a reasonably accurate reflection of the ratio of renal plasma flows and that there were no major changes in the renal plasma flows of the control kidneys. With the reservation necessary under the circumstances, it would appear that very marked changes in the concentration of the urine occurred when the renal blood flow of the experimental kidney was negligibly reduced.

DISCUSSION

The experiments which have been described indicate that circumstances associated with constriction of one renal artery cause a rise in the concentration of the urine produced by the affected kidney and that the urine from this kidney may become hypertonic to plasma. Participation of pituitary antidiuretic hormone in the phenomenon can be excluded with reasonable certainty for the following reasons: Normal water diuresis characteristic of the absence of antidiuretic hormone was maintained throughout by the contralateral con-

[7] In the experiment in Table III, chloride excretion by the control kidney did not exceed 2 μEq. per minute, and sodium excretion barely exceeded 1 μEq. per minute. The data have therefore been omitted.

[8] It has been reported that when PAH and glucose are contained in the same infusion flask there is a chemical change in the PAH which reduces its renal extraction (16). Through an oversight, in some of the present experiments PAH and glucose were mixed in the same flask. However, falls in PAH clearance were observed when no glucose was administered (Table II) and when the PAH and glucose were administered in separate infusions (Table I).

trol kidney. This maximal water diuresis was indicated by the low concentration of the urine when solute excretion was low and by the high rate of excretion of solute-free water (free water clearance) (3) when solute excretion was high. The possibility that the kidney with constricted renal artery was sensitized to amounts of circulating ADH insufficient to produce detectable effects on the normal kidney may be excluded since 1) the administration of amounts of ADH inadequate to affect the normal kidney had no effect on the experimental kidney, 2) the infusion of moderate amounts of ADH produced more change in the concentration of the urine from the control than that from the experimental kidney, and 3) during periods of apparent endogenous ADH secretion (*e.g.*, the first two periods shown in Figure 6) the effect was similar. From these considerations it may be concluded that ADH is not essential to the production of hypertonic urine. These conclusions are in accord with those of del Greco and de Wardener (8). However, in several of the experiments reported by del Greco and de Wardener the control urine concentrations were relatively high and the free water clearances rather low and in these studies the kidneys were, under the control conditions, unresponsive to the effects of ADH. It is therefore impossible to exclude some participation of the hormone in the changes observed when a balloon was inflated in the aorta. The continued normal function of the control kidney while hypertonic urine was produced by the experimental kidney in the present experiments is fully in accord with their conclusions.

It is the presumption that, in accord with the rationale of the experiments, the effects observed are the result of reduction of glomerular filtration rate and the consequent changes in the distribution of solute and water reabsorption along the length of the nephrons. However, the method used produces effects other than reduction of glomerular filtration and it is necessary to consider whether one of these may be directly involved in the experimental results. The most obvious possibility would be an effect mediated by changes in the renal blood flow. Anoxic impairment of transport by the renal tubules might lead to inability to dilute the urine by a process dependent upon solute extraction. However, under such conditions one would expect the composition of the urine to approach that of glomerular filtrate and the fraction of filtered solute excreted in the urine to increase. The changes observed were in the opposite direction: the fall in urine flow *and* solute excretion was out of proportion to the reduction in the rate of glomerular filtration, especially so when mannitol was not included. Furthermore, the concentrations of sodium and chloride were generally vanishingly small in the more concentrated urine from the experimental kidney and these are the solutes presumably specifically involved in the dilution process. Impairment of the dilution process alone cannot, of course, account for the production of urine hypertonic to plasma.

Anoxic kidney slices have been found to take up water and swell (17, 18). Although the mechanism involved in this phenomenon is disputed and it is uncertain whether it involves the simple uptake of water (17) which might increase the concentration of the surrounding medium, or the uptake of solute and water in the proportions in which they occur in the medium (18) which would not change the osmolality of the latter, for present purposes only the uptake of water alone would be pertinent. Such an uptake of water could, presumably, increase the concentration of fluid in the tubule lumen above that characteristic of the rest of the body. However, the process is self-limited since the cells could take up water only until their contents had been diluted to the same concentration as the general body fluids and plasma. On such a basis it is difficult, if not impossible, to account for the continuous formation of hypertonic urine over long periods (*e.g.*, Table I). Furthermore, one would have to make the highly improbable assumption that the "hypertonic cells" selectively take up water from their luminal surfaces deriving the excess water only from the urine while remaining impermeable on their other surfaces where they are exposed to far larger volumes of fluid of normal composition.

Actually it seems probable that the changes in renal blood flow associated with major alterations in urine concentration were not large enough to produce significant renal anoxia, particularly since plasma flow and filtration rate are known to be markedly increased in the dog under conditions of water loading such as those used in these studies.

It seems reasonable, therefore, to attribute the

effects of inflation of the renal artery cuff to the reduction of glomerular filtration. Further, the data are in accord with the interpretation that the reduction is due to diminution in the volume filtered in all or very nearly all the nephrons rather than to a selective cessation of filtration in a group of nephrons.[9] If the latter were the case, it would be necessary to postulate that when a 20 per cent reduction of glomerular filtration leads to production of hypertonic urine, the 20 per cent of nephrons, function of which had been abolished, had been responsible for the output of 80 to 85 per cent of the urine water, almost 99 per cent of the excreted sodium and chloride and, from the results of more recent experiments (21), none of the excreted potassium.

The most important effect of reduced glomerular filtration is probably a reduction in the amount of sodium and chloride reaching and reabsorbed by the distal portions of the nephron. The rapid falling off of sodium and chloride excretion as the filtration rate is reduced is in accord with the view that the reabsorption of these ions becomes essentially complete farther and farther up the tubules. Since only the sodium reabsorbed in the more distal portions of the nephron is involved in the process of urinary dilution (3), a limitation is specifically placed on that part of the sodium and chloride reabsorption which contributes to the formation of solute-free water. In addition, it cannot be assumed that the tubule is, even in the absence of antidiuretic hormone, totally impermeable to water. Experiments showing that the rate of excretion of solute-free water increases with increasing solute excretion (22) indicate that the diffusion gradient for water has an important effect on the amount of water, initially freed by solute reabsorption, that escapes from the tubule. Because of the reduced delivery of sodium and chloride to the diluting segment, less water is freed of solute and because of the small amount of residual solute, an increased fraction of the solute-free water escapes from the tubule lumen. Thus, a greatly reduced volume of fluid including only a minimal amount of solute-free water is delivered to the final segment of the nephron presumed to be responsible for the production of hypertonicity (3, 23). Removal of only a small volume of the residual water is then necessary for the elaboration of a urine hypertonic to plasma.

The effect of increased excretion of non-reabsorbable solute (mannitol) can be viewed as twofold: 1) in increasing the amounts of sodium and chloride escaping reabsorption in the upper portions of the nephron (24, 25), and 2) in exerting a non-specific osmotic effect opposing the escape of water, initially freed of solute, from the tubule (22).

Although urines considerably more concentrated than plasma were produced in these experiments, the urine osmolality never approached the maximal levels which can be obtained with ADH at low rates of solute excretion. At the same time, the urine volume was never less than the osmolal clearance by more than a few tenths of a milliliter per minute (*i.e.*, $T^c_{H_2O}$ was always less than 0.3 ml. per minute). It is unlikely that these limitations could be due to either a limiting osmotic gradient or to a limited absolute capacity for water transport by the concentrating mechanism. These have been considered to be the important parameters for urinary concentration in the presence of maximally effective amounts of ADH (3). It seems more probable that the limited effectiveness of the concentrating mechanism is attributable to inefficiency of its operation in the absence of ADH.

The results of these experiments are fully in accord with the view that the sole action of ADH in the kidney is on the permeability of the renal tubule to the movement of water along osmotic gradients. Its role in the production of hypertonic urine can be interpreted as 1) facilitating the diffusion of water out of the tubule as solute is removed in the distal segment, thus assuring the delivery of an essentially isotonic fluid to the concentrating segment, and 2) (assuming the mechanism for producing the final hypertonicity to op-

[9] Experiments very similar in design but differing in the surgical technique from those reported here have been reported by Leaf, Kerr, Wrong, and Chatillon (19). In their studies the experimental kidney was explanted and the renal artery enclosed in a skin cuff. Marked changes in the rate of glomerular filtration in their experiments were not accompanied by any systematic changes in urine concentration. The results in their experiments and in our own were each so internally consistent that it must be presumed that the difference is attributable to the difference in technique. Dr. Leaf has suggested that the changes of glomerular filtration rate in their experiments most likely were due to cessation of the function of certain groups of nephrons (20).

erate independently) to increase the accessibility of the concentrating mechanism to the water in the tubule. The results are not easily explained in terms of a urinary dilution mechanism based on secretion of water into the urine.

Whatever the specific chain of events involved in the studies reported here, it is obvious that a number of possibilities other than simple changes in the level of circulating ADH must be considered when changes in urine concentration and in the rate of elimination of water loads are encountered. Changes in the amount of sodium reaching the diluting segment or in the capacity of that segment to reabsorb ions can account for major changes in the rate of excretion of water and even for the production of hypertonic urine.

On the other hand, it cannot be assumed that simple reduction of the rate of glomerular filtration in both kidneys on a chronic basis will necessarily result in the phenomena dealt with in these experiments since the continued presence of a normal kidney in these experiments may, as in the studies of Mueller, Surtshin, Carlin, and White (*26*), prevent compensatory changes which would restore diuresis to normal levels.

SUMMARY

In water-loaded dogs compression of one renal artery leads to a rise in the concentration of the urine produced by the affected kidney. It is thus possible for one kidney to produce urine hypertonic to plasma while the other kidney excretes water at a maximal rate. The changes were shown not to be due to sensitization of the experimental kidney to the action of minimal amounts of ADH. The important factor concerned is believed to be the reduction of the rate of glomerular filtration and thus the filtered load of sodium and chloride in each nephron. Since reduced amounts of sodium and chloride reach the segment responsible for dilution of the urine, the extent to which the urine can be diluted by abstraction of these ions is limited. Diffusion of water from the diluted urine through the relatively impermeable tubule results in delivery of a small volume of only slightly diluted urine to the concentrating segment. The concentrating mechanism, operating essentially without dependence on ADH, is able to remove enough water to render the urine hypertonic to plasma. The role of ADH is interpreted as that of increasing the permeability of the diluting and possibly the concentrating segments to the movement of water along osmotic gradients. Factors having to do with the load and disposition of sodium as well as ADH must be considered in the interpretation of changes in urine concentration.

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THE DESTRUCTION OF RED CELLS BY ANTIBODIES IN MAN. I. OBSERVATIONS ON THE SEQUESTRATION AND LYSIS OF RED CELLS ALTERED BY IMMUNE MECHANISMS[1]

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Much of our present knowledge of the pathologic physiology of the acquired hemolytic anemias originated from observations of the fate of heterologous red cells in immunized animals and of the syndromes produced in animals by injections of heterologous anti-red cell sera (1–17). The frank hemolysins present in anti-red cell sera seemed to provide a complete explanation for the experimental findings, but it was early stressed by Muir and M'Nee (6) and by Banti (8) that the hemolytic action of hemolysin-containing sera was greater in extent and slower in evolution when injected into the intact animal than when added to red cells *in vitro*. It was also pointed out by Banti (8) and by others (12, 14) that anti-red cell sera produced spherocytosis of red cells *in vivo* but failed to do so *in vitro*. In humans it was proposed that spontaneous hemolysins may provoke the clinical picture of acquired hemolytic anemia (5, 9, 10), particularly when associated with high titers of cold agglutinins (18, 19). However, clinical experience with acquired hemolytic anemia as well as with blood transfusion reactions and erythroblastosis fetalis has indicated that these hemolytic syndromes are generally not associated with hemolysins demonstrable *in vitro* (20, 21); and the injection into animals (14, 17, 22) or into man (23–26) of antibodies which agglutinate but do not directly hemolyze red cells *in vitro* may reproduce the full picture of clinical hemolytic disease. Indeed, a number of investigators have observed the formation of intravascular red cell agglutinates *in vivo* after injection of heterologous or homologous immune serum (4, 14, 15, 24, 27). In many patients suffering from hemolytic reactions, in almost all infants afflicted with erythroblastosis fetalis, and in most patients with acquired hemolytic anemia, it is not even possible to demonstrate "complete" agglutinins. In these conditions the antibodies (actual or presumed) are agglutinins only in the sense that they "sensitize" red cells to the agglutinating effect *in vitro* of antiglobulin (Coombs) serum, of sufficient concentrations of plasma proteins or of certain other large anisometric anionic molecules (28–30). Although the hemolytic effect of hemolysins *in vivo* may be inferred from their manifest effects *in vitro*, the mechanism of the hemolytic action *in vivo* of agglutinating and of sensitizing antibodies has remained obscure. In the present study, use was made of the Cr^{51} method for labelling small volumes of red cells for red cell survival studies (31, 32) and for determining the sites of red cell sequestration in living subjects (33). The subjects on whom observations were made included normal adults who had previously suffered accidental isoimmunization through transfusion or pregnancy. Some of these findings have been presented in preliminary form elsewhere (34–36).

METHODS

Techniques employing Cr^{51}. Cr^{51} in the form of $Na_2Cr^{51}O_4$[2] was employed for red cell labelling (31) as adapted for studies of red cell survival by Ebaugh, Emerson, and Ross (32). Volumes of sodium chromate solution possessing an activity of 100 to 150 microcuries of Cr^{51} were added to 30 to 40 ml. of freshly-drawn blood and 12 ml. of ACD solution in sterile 100-ml. siliconized flasks. Following 45 minutes of gentle continuous agitation of the flasks at room temperature, their contents were centrifuged in sterile tubes and the supernatant solutions were removed. The packed cells were washed once in sterile isotonic saline, prior to their resuspension in saline to a final volume of about 30 ml. The suspensions were injected in a period of about one minute.

Samples of blood were drawn periodically from the

[1] This investigation was supported in part by a grant from the Helen Hay Whitney Foundation, and by Research Grants RG3507(C3) and H1227 from the National Institutes of Health, Public Health Service.

[2] "Rachromate" of high specific activity, Abbott Laboratories, North Chicago, Illinois.

subject in each experiment into saline-wetted syringes and then were divided into two parts. One part, which was received into a bottle containing dry "balanced" oxalate, was lysed by repeated freezing and thawing to provide samples for measuring whole blood radioactivity. The other part, received into one-tenth of its volume of 3 per cent sodium citrate solution, was centrifuged to provide plasma for determination of plasma radioactivity. In each case the plasma was re-centrifuged prior to sampling. The radioactivity of 3-ml. samples of whole blood, plasma, urine, and the Cr^{51} standards, was determined in a well-type scintillation counter.

The distribution of radioactivity over the hearts, livers and spleens of subjects receiving injections of Cr^{51} was determined with a directional scintillation counter. The methods employed and the interpretation of the data obtained with these procedures are described elsewhere (33).

Techniques employing Fe^{59}. Walsh, Thomas, Chow, Fluharty, and Finch (37) have observed that human reticulocytes can incorporate Fe^{59} into hemoglobin *in vitro*. Accordingly, blood samples were utilized from several patients with reticulocyte-rich blood for observations of Fe^{59}-labelled reticulocyte survival *in vivo*. A complex between Fe^{59} and human iron-binding protein was obtained by slowly adding dilute $Fe^{59}Cl_3$ [3] to the subject's sterile heparinized plasma in an amount calculated not to exceed the subject's plasma iron-binding capacity. One volume of such Fe^{59}-labelled plasma was then incubated at 37° C. with one volume of reticulocyte-rich red cells for from one to two hours in sterile siliconized flasks. Since separate studies indicated that from 0.5 to 1 microgram of Fe^{59} was taken up by each cubic centimeter of reticulocytes under these conditions, the volume of red cells used was selected to contain between 5 and 10 ml. of reticulocytes in order to provide a suitable amount of radioactivity. Because at the end of the incubation period most of the Fe^{59} still remained in the plasma, the labelled red cells were washed in sterile isotonic saline five times prior to their resuspension in saline at a final volume of 50 ml. They were then injected intravenously.[4] In these suspensions over 99 per cent of the radioactivity was in the red cells; of this, between 50 and 90 per cent, generally about 70 per cent, was incorporated into hemoglobin as determined by filter paper electrophoresis [5] and the remainder was present in the red cell membrane. None of the radioactivity was elutable from the labelled red cells during incubation in fresh plasma for 24 hours at 37° C. The techniques of determining and of presenting data on blood and body surface radioactivity subsequent to the injection of Fe^{59} were similar to those employed with Cr^{51}-labelled red cells (33).

Techniques involving antisera and red cell sensitization. Human antisera were obtained from persons who had suffered primary isoimmunization against the C or D antigens of the Rh system as a result of transfusion or pregnancy. These donors had subsequently been hyperimmunized by small injections of incompatible red cells. Serum or plasma drawn from these donors was confirmed as sterile by appropriate bacterial culture methods.

The sensitization of normal red cells was carried out by incubating together for one hour at 37° C. a mixture of one volume of packed type D red cells and two volumes of an appropriate antiserum diluted with an equal amount of saline. After incubation the cells were washed twice prior to injection. Blood from cases of erythroblastosis fetalis [6] was obtained in 30-ml. amounts via the umbilical vein prior to the first transfusion in exchange transfusions. In all experiments, the labelled, sensitized, washed red cell suspensions used were examined for hemoglobin and Cr^{51} concentrations, for agglutination by Coombs serum and by polyvinylpyrrolidone (P.V.P.) (30) and for possible bacterial contamination.

Miscellaneous techniques. Plasma hemoglobin levels were determined by a photoelectric adaptation of the benzidine technique (39), using the same specimens employed for measuring plasma Cr^{51} levels. The Coombs (antiglobulin) test (40) was performed using the tube technique with potent anti-human serum rabbit serum prepared as described by Emerson, Franklin, and Lowell (41). The technique of the "P.V.P. test" has been described elsewhere (30). Osmotic (42) and mechanical (43) fragilities of red cells were determined in several experiments. The Cr^{51} activity of dog tissue specimens was determined after these tissues had been digested with 10 per cent NaOH and "homogenized" in a Waring blendor. The total protein concentration of heparinized dog plasma was measured by the micro-Kjeldahl method (44) and the albumin concentration by the dye method (45). Serum bilirubin levels were determined by the photoelectric method (46, 47) and were recorded as the one-minute or prompt direct-reacting bilirubin ("direct bilirubin") and as total bilirubin.

Human subjects. The "normal" human subjects studied were generally elderly men with minor illnesses, in whom no diseases of the hematopoietic or reticulo-endothelial systems were believed to be present.

Animal subjects. Adult male mongrel dogs were anesthetized with Nembutal® two hours prior to the injection of autogenous Cr^{51}-labelled red cells and remained unconscious until sacrificed. A few minutes prior to sacrifice of each animal 50 mg. of heparin was injected intravenously. Agglutination of canine red cells *in vitro* was produced by exposing the washed labelled red cells to

[3] Abbott Laboratories, North Chicago, Illinois.

[4] In compliance with the definition of tracer doses of radioactive isotopes in adult man as being that quantity of radioactive material emitting radiation in an amount less than a total of 1 rep (38), the maximum dose of Fe^{59} given should not exceed 0.2 microcuries per kilogram body weight. In the studies reported here from 4 to 6 microcuries of Fe^{59} were injected.

[5] Since serum iron-binding protein and hemoglobin migrate together in the commonly used alkaline barbital buffer, it is advisable, for the separation of these proteins, to employ a buffer with a pH intermediate between the isoelectric points of the proteins. For this purpose a pH 6.4, 0.1 molar phosphate buffer was selected.

[6] We are indebted to Dr. Jane Desforges for assistance in procuring several of these specimens.

trivalent chromium ($CrCl_3$), one of many multivalent cations which agglutinate the red cells of various species (48). "Sensitization" of canine red cells to agglutination by Coombs serum was achieved by incubating specimens of whole blood with a subagglutinating amount of trivalent chromium ($CrCl_3$).[7] The extent of "sensitization" was determined with a specially prepared Coombs serum (anti-dog serum rabbit serum) and with P.V.P. "Spleen blood" was obtained by deeply incising the splenic parenchyma after clamping the splenic pedicle and rapidly excising the organ. The effluent blood was allowed to flow directly into test tubes containing heparin.

[7] The manner in which certain multivalent cations such as Cr^{+++} "sensitize" red cells to the agglutinating action of Coombs serum is presented elsewhere (48). These metals are entirely inactive in this respect when in anionic complexes such as chromate.

RESULTS

Sequestration of red cells altered by antibodies

Agglutinating antibodies. Several observations were made on the fate of ABO-incompatible normal red cells in subjects in whom normal agglutinin titers were present and in whom *low con-*

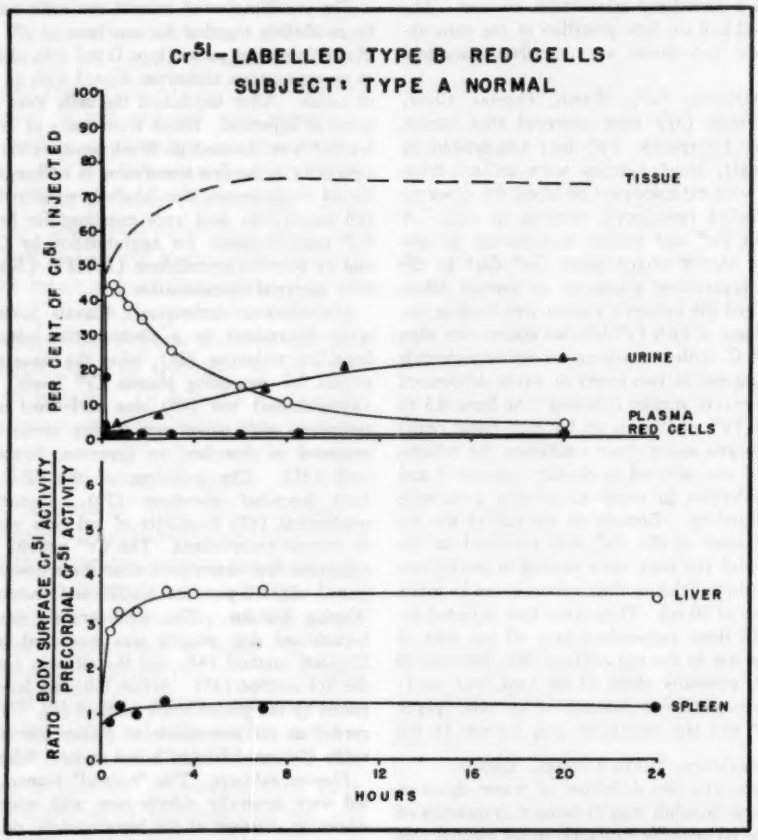

FIG. 1. DESTRUCTION OF TYPE B RED CELLS IN A NORMAL TYPE A RECIPIENT WHOSE SERUM CONTAINED ISOAGGLUTININS AND WEAK ISOHEMOLYSINS

Virtually all of the red cell Cr^{51} activity disappeared from the recipient's circulation within two minutes after injection of these incompatible red cells, with the prompt appearance of high levels of Cr^{51} (and of hemoglobin) in the plasma. Approximately 25 per cent of the injected Cr^{51} appeared in the urine within 24 hours of the injection. That radioactivity not present in red cells, plasma, or urine was presumably in "tissue." The "organ" distribution of tissue Cr^{51} is depicted in the lower portion of this figure, where the prompt and comparatively high radioactivity of the liver is apparent.

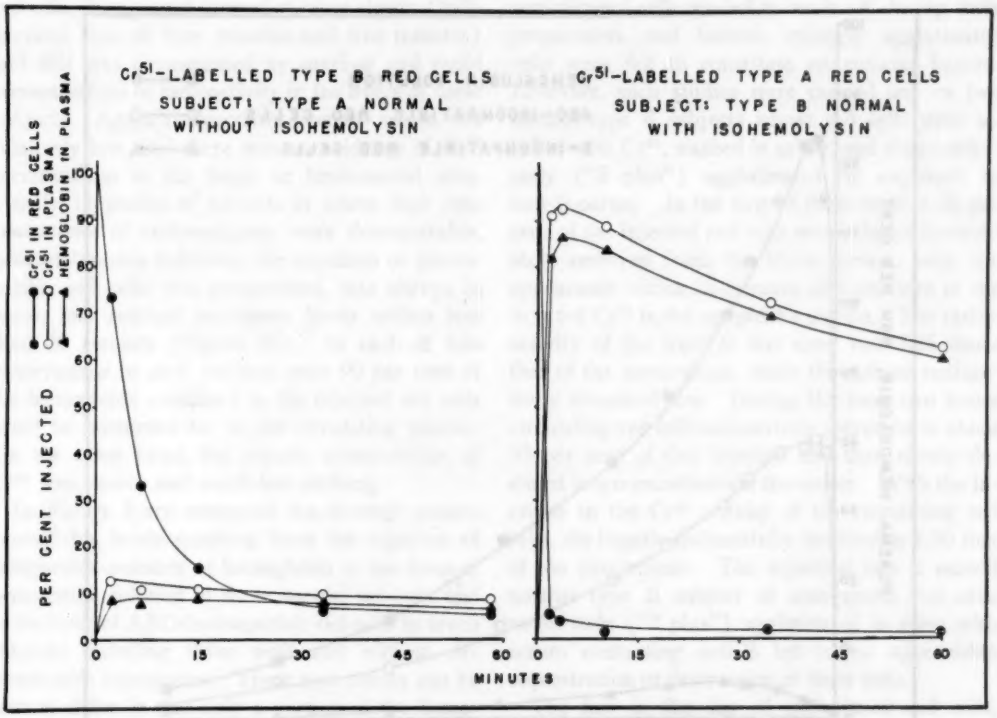

FIG. 2. COMPARISON OF THE PATTERN OF DESTRUCTION OF NORMAL RED CELLS BY ISOAGGLUTININS AND BY ISOHEMOLYSINS

a. Type B red cells (left above) injected into a normal type A subject with a 1:64 titer of anti-B agglutinin, but without anti-B hemolysin demonstrable *in vitro*, were removed rapidly (half-disappearance time: 4 minutes), with the appearance of immediate but relatively low levels of Cr^{51} and of hemoglobin in the plasma. There was a heavy Cr^{51} accumulation by the liver. After 60 minutes the relative radioactivity values were: liver/precordium, 7.13; spleen/precordium, 1.86. After 18 hours when the plasma radioactivity had declined to low levels the liver/precordium ratio was unchanged.

b. The injection of type A red cells (right above) into a normal type B subject with a 1:256 titer of anti-B agglutinin, whose undiluted fresh serum caused 2 plus hemolysis of type B red cells *in vitro*, produced immediate disappearance of 90 per cent of the injected Cr^{51}-labelled red cells from the blood stream, and comparatively high levels of Cr^{51} and of hemoglobin in the plasma. In this subject hepatic radioactivity was less striking and slower to appear. After 60 minutes the relative radioactivity values were: liver/precordium, 1.72; spleen/precordium, 1.02. After 18 hours, when plasma radioactivity had declined to low levels, the liver/precordium ratio was 2.94.

centrations of isohemolysins were demonstrable *in vitro*.[8] A representative observation is portrayed in Figure 1. The Cr^{51}-labelled red cells are seen largely to disappear from the circulation within two minutes of the injection, in association with the abrupt appearance of high levels of radioactivity over the patient's liver, but not over the spleen, thorax, or lumbosacral areas. This appearance of high hepatic Cr^{51} levels occurred considerably faster than when Cr^{51}-labelled hemoglobin was injected intravenously (33). Immediately after injection of the labelled ABO-incompatible cells, both Cr^{51} and hemoglobin appeared in the patient's plasma, reached maximum values

[8] In evaluating the activity *in vitro* of isohemolysins or other antibodies it is important to employ the same ratio of serum to cells which is to be studied *in vivo*. In normal subjects complete hemolysis often occurred only when this ratio was greater than 500:1 (the ratios achieved *in vivo* averaged about 200:1), whereas none occurred at ratios of less than 100:1 or 200:1. Presumably a critical concentration of hemolysin must be absorbed on each cell, and a zone of "antigen excess" may obscure results derived from conventional *in vitro* serum-cell ratios of 100:1 or less.

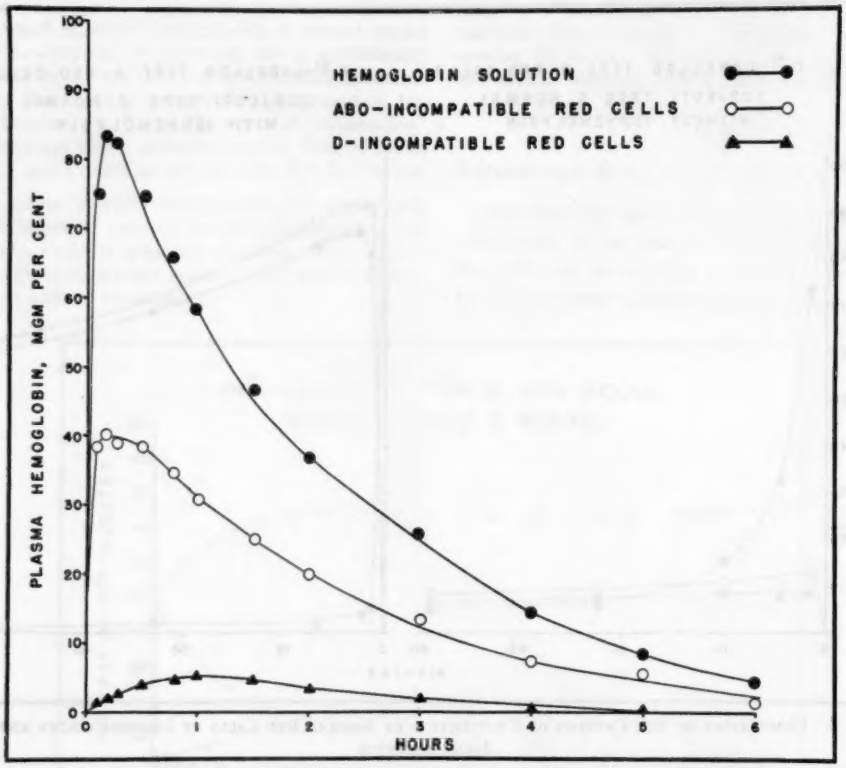

FIG. 3. PLASMA HEMOGLOBIN LEVELS IN NORMAL SUBJECTS FOLLOWING THE RAPID INTRAVENOUS INJECTION OF VARIOUS PREPARATIONS CONTAINING APPROXIMATELY 3 GM. OF HEMOGLOBIN

The points on each curve represent the average of data obtained from several subjects. The observed plasma hemoglobin values were corrected for pre-injection levels. Injection of 2.8 Gm. of hemoglobin in solution into each of three subjects produced no hemoglobinuria or hemosiderinuria; following a mixing period of 15 minutes, the plasma hemoglobin levels diminished exponentially with a half-disappearance time of about 100 minutes. Injection of ABO-incompatible red cells containing a similar amount of hemoglobin into seven normal subjects, two of whom had no demonstrable hemolysins against the injected red cells, caused less hemoglobinemia. Following a longer mixing period of about 35 minutes, the plasma hemoglobin levels also declined exponentially, with a half-disappearance time of about 90 minutes. Injection of comparable amounts of type D red cells into three subjects previously immunized against the D factor and possessing high titers of incomplete anti-D antibodies produced much smaller and more gradual rises in plasma hemoglobin levels (see Figure 12 for the results in individual patients).

of about 40 per cent of the injected Cr^{51} and hemoglobin within five or six minutes, and gradually diminished thereafter over a period of several hours. A fairly prominent excretion of Cr^{51} was found in the urine but hemoglobinuria was not detected. Observations were made on the fate of ABO-incompatible red cells injected into two subjects having normal isoagglutinin titers but in whom *no isohemolysins* were demonstrable *in vitro* at any serum-to-cell ratio despite the addition of fresh complement. In these subjects the Cr^{51} and hemoglobin contents of the circulating plasma rose immediately to approximately 10 per cent of the amounts injected and did not thereafter exceed this comparatively low level (Figure 2a). As compared to the first group of subjects, the labelled

red cells disappeared somewhat more slowly (half-survival time of four minutes and five minutes) and this was accompanied by marked and rapid accumulations of radioactivity in the livers of these subjects. Again, radioactivity over the spleen was relatively low, and there was no evidence of Cr^{51} accumulation in the lungs or lumbosacral area. Finally, in studies of subjects in whom *high concentrations of isohemolysins* were demonstrable, hemoglobinemia following the injection of incompatible red cells was pronounced, was abrupt in onset, and reached maximum levels within less than 60 seconds (Figure 2b). In each of two observations in such subjects over 90 per cent of the hemoglobin contained in the injected red cells could be accounted for in the circulating plasma. On the other hand, the hepatic accumulation of Cr^{51} was slower and much less striking.

In Figure 3 are compared the average plasma hemoglobin levels resulting from the injection of comparable amounts of hemoglobin in the form of hemoglobin solution in three normal subjects and in the form of ABO-incompatible red cells in seven subjects including those with and without demonstrable hemolysins. These two curves can be seen to differ in that only a portion of the hemoglobin derived from ABO-incompatible red cells was released into the circulation (average: 42 per cent) and that there was a more prolonged hemoglobin "mixing" period after the injection of incompatible red cells than was the case when hemoglobin solution was directly injected.

Observations in human subjects of the rate of removal of red cells agglutinated *in vitro* by ABO-incompatible serum prior to their injection were largely frustrated because antibodies on weakly-agglutinated cells tended to wash off during their preparation, and because strongly agglutinated cells were felt to constitute an embolic hazard. However, such studies were carried out on two normal type B subjects whose red cells were labelled with Cr^{51}, washed in saline, and then moderately ("2 plus") agglutinated by exposure to anti-B serum. In the first of these studies 26 per cent of the injected red cells were almost immediately removed from the blood stream, with the appearance within 25 minutes of 6 per cent of the injected Cr^{51} in the recipient's plasma. The radioactivity of the liver at this time was 2.25 times that of the precordium, while the splenic radioactivity remained low. During the next two hours circulating red cell radioactivity increased to about 93 per cent of that injected and then slowly declined in a normal fashion thereafter. With the increase in the Cr^{51} activity of the circulating red cells, the hepatic radioactivity declined to 1.50 that of the precordium. The injection into a second normal type B subject of autogenous red cells moderately ("2 plus") agglutinated *in vitro* with serum containing anti-B led to no appreciable sequestration or destruction of these cells.

The fate in the dog of autogenous red cells, moderately-heavily ("3 plus") agglutinated by $CrCl_3$ is presented in Table I. Here is depicted a marked retention of such red cells by the animal's lungs, with some uptake by the liver, and very little by the spleen.

Non-agglutinating ("incomplete") antibodies. Four subjects with high titers of incomplete anti-D antibody were injected with Cr^{51}-labelled type D red cells. Type D red cells exposed to the sera of these subjects were sensitized to the antiglobu-

TABLE I

Organ distribution in two dogs of Cr^{51} derived from autogenous red cells previously labelled with $Na_2Cr^{51}O_4$ and exposed to a multivalent metallic cation

Dog	Red cell suspension: Agglutination in saline	Red cell suspension: Coombs test	Method of preparation	Distribution of Cr^{51} 2 hours after injection, %: Blood	Liver	Spleen	Lung	Kidney	Total
S-2	3+	0*	Red cells in saline + $CrCl_3$	2.6	8.2	0.3	60.2	0.1	71.4
S-4	0	2+	Red cells in serum + $CrCl_3$	62.9	7.8	25.9	0.3	0.1	97.0

* That is, the intensity of agglutination was not augmented by an antiserum developed against dog serum in rabbits.

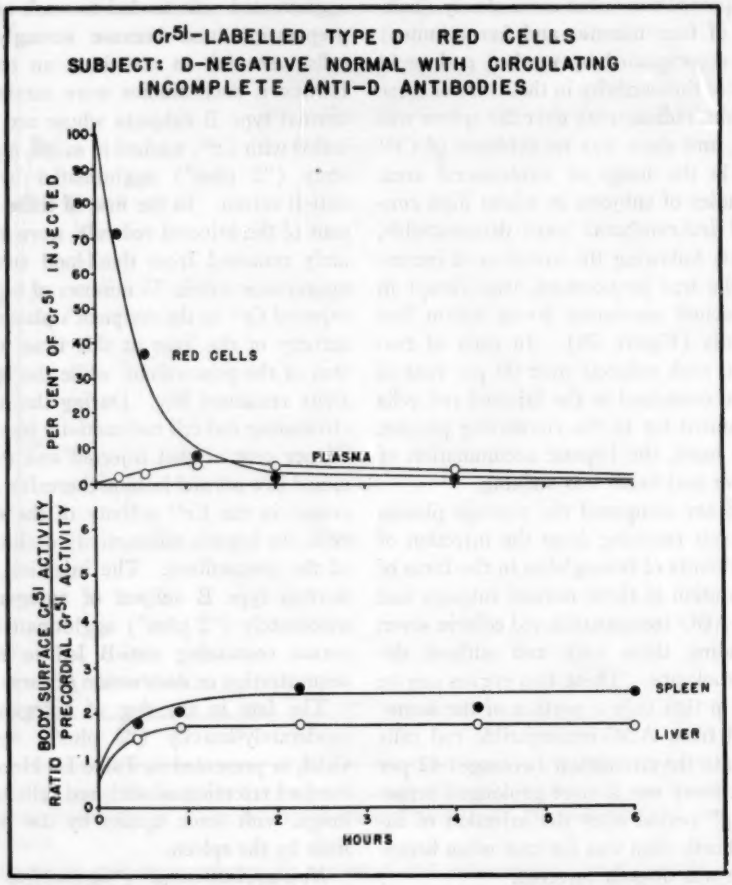

FIG. 4. DESTRUCTION OF Cr^{51}-LABELLED TYPE D RED CELLS IN AN OTHERWISE NORMAL D-NEGATIVE SUBJECT WHOSE SERUM CONTAINED INCOMPLETE ANTI-D ANTIBODIES

Following intravenous injection, half the red cell Cr^{51} activity disappeared from the patient's peripheral blood in 21 minutes, while plasma Cr^{51} levels climbed to a maximum level of only 4.5 per cent of the injected dose about one hour after injection. A moderate accumulation of Cr^{51} in the subject's spleen, and a slightly smaller accumulation by the liver, occurred simultaneously with the disappearance of red cell Cr^{51}.

lin reaction but were not agglutinated by resuspension in undehydrated native plasma. A representative study is portrayed in Figure 4, in which is seen the rapid removal of Cr^{51}-labelled red cells from the circulation. The half-survival time of type D red cells in these hyperimmunized subjects ranged from 8 to 24 minutes, and averaged 14 minutes. In these studies an associated moderate uptake of Cr^{51} by the spleen and, to a lesser extent, by the liver was observed. Little or no Cr^{51} or hemoglobin appeared in the plasma when the injected red cells contained about 1 Gm. of hemoglobin, and both levels were low even when the hemoglobin content exceeded 6 Gm.[9] and when the quantity of red cells destroyed within the first

[9] The injection of approximately 10 ml. of type D red cells into these subjects uniformly produced moderate to severe febrile reactions analogous to those produced by the injection of bacterial pyrogens. This also occurred when similar amounts of previously-sensitized red cells were given to normal subjects. The mechanisms of this reaction and of the associated leukopenia are now under study (49).

10 minutes exceeded that of the ABO incompatibility studies described above. When these four subjects received injections of type D red cells containing 3.3 ± 0.5 Gm. of hemoglobin, the maximum levels of Cr^{51} and of hemoglobin in the plasma were attained about one hour after injection, at which time only about 5 per cent of the injected hemoglobin was present in the circulating plasma.

When the recipient's serum contained agglutinating as well as incomplete anti-C antibodies (Figure 5), the sequence of events was quite similar to that of recipients with only incomplete antibodies (Figure 4), except that red cell sequestration was somewhat more rapid and hepatic radioactivity was more pronounced. Again, plasma hemoglobin and Cr^{51} levels increased slowly to reach maximum levels about one hour after the injection.

Additional studies were made on the destruction of Cr^{51}-labelled D-positive normal red cells which had been sensitized *in vitro* with incomplete anti-D antibodies prior to their reinjection into the donor or into an ABO-compatible normal subject.

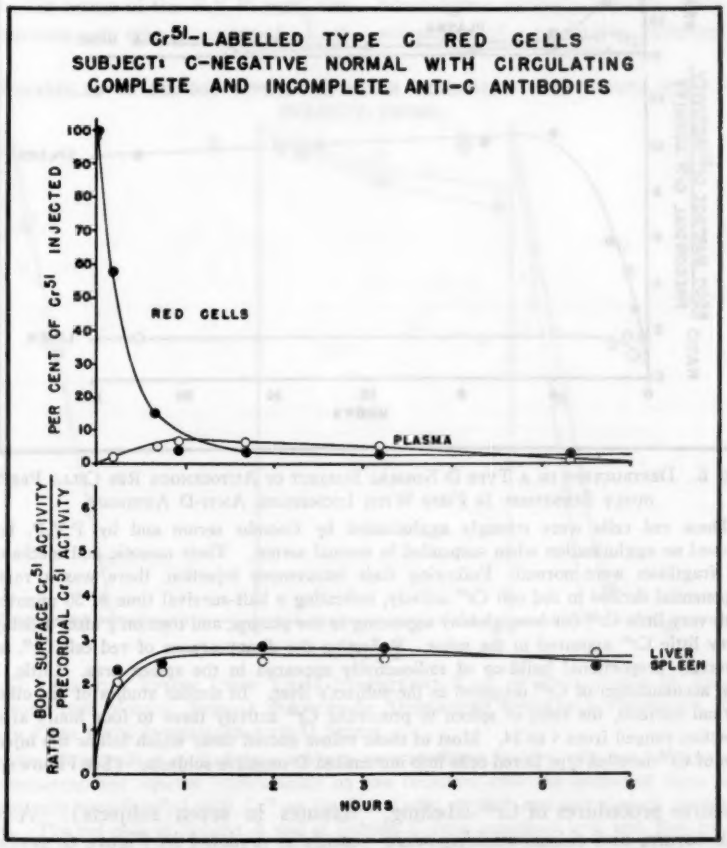

FIG. 5. DESTRUCTION OF TYPE C RED CELLS IN AN OTHERWISE NORMAL C-NEGATIVE SUBJECT WHOSE SERUM CONTAINED BOTH COMPLETE AND INCOMPLETE ANTI-C ANTIBODIES

Following their intravenous injection half of these red cells were removed from the blood stream within eight or nine minutes, with the plasma Cr^{51} reaching a maximum value of only 6 per cent of the injected dose about one hour later. The liver and spleen were about equally active in sequestering these red cells.

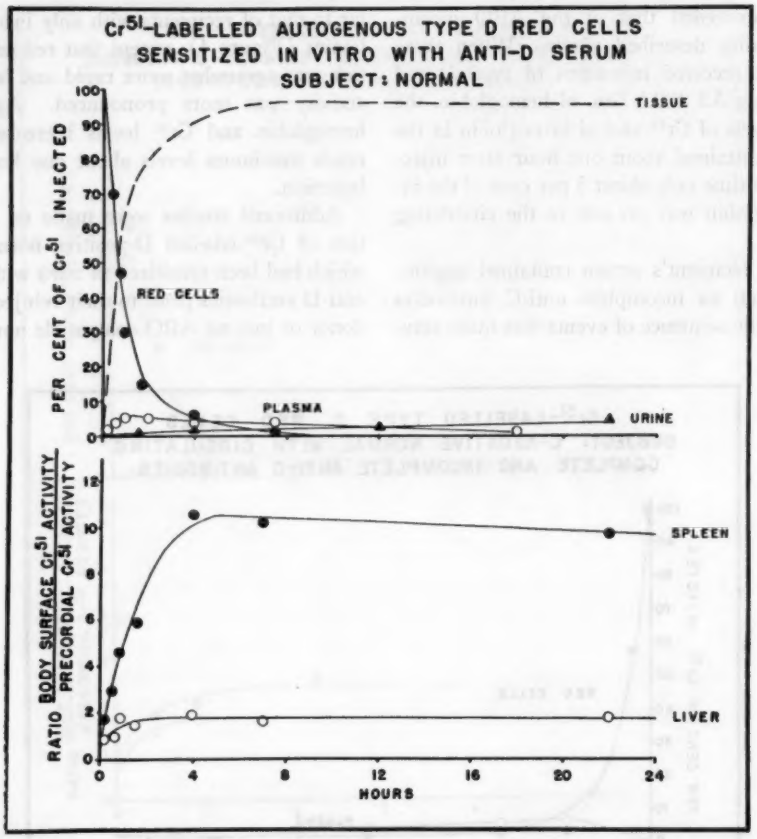

FIG. 6. DESTRUCTION IN A TYPE D NORMAL SUBJECT OF AUTOGENOUS RED CELLS PREVIOUSLY SENSITIZED *In Vitro* WITH INCOMPLETE ANTI-D ANTIBODY

These red cells were strongly agglutinated by Coombs serum and by P.V.P. but showed no agglutination when suspended in normal serum. Their osmotic and mechanical fragilities were normal. Following their intravenous injection, there was a rapid exponential decline in red cell Cr^{51} activity, indicating a half-survival time of 30 minutes, with very little Cr^{51} (or hemoglobin) appearing in the plasma, and then only after a delay. Very little Cr^{51} appeared in the urine. Reflecting the disappearance of red cell Cr^{51}, an inversely proportional build-up of radioactivity appeared in the spleen area. Little, if any, accumulation of Cr^{51} occurred in the subject's liver. In similar studies of six other normal subjects, the ratio of spleen to precordial Cr^{51} activity three to four hours after injection ranged from 4 to 14. Most of these values exceed those which follow the injection of Cr^{51}-labelled type D red cells into immunized D-negative subjects. (See Figure 4.)

When the successive procedures of Cr^{51}-labelling, exposure to anti-serum, and saline washing were performed with D-negative red cells their subsequent survival *in vivo* was not affected. When, however, D-positive red cells were incubated with potent incomplete anti-serum, half of the injected sensitized cells were removed from the subjects' circulation in from 18 to 40 minutes (average, 26 minutes in seven subjects). A representative study is depicted in Figure 6, revealing the relatively rapid disappearance of labelled red cells associated with only slight increases of Cr^{51} and of hemoglobin in the plasma. Very little Cr^{51} appeared in the urine. During the first 15 or 20 minutes after the injection of the sensitized but originally unagglutinated red cells, small numbers of

red cell agglutinates and chains were frequently visible in the recipient's peripheral blood. These could be most readily visualized by diluting the blood 200-fold in saline and examining the freely-moving cell suspension microscopically under a cover-slip with the "high dry" lens. Characteristic of, and concomitant with, the red cell destruction was a heavy build-up of Cr^{51} by the spleen, with little, if any, evidence of Cr^{51} accumulation by the liver. In three ABO-compatible, unimmunized D-negative subjects, D-positive red cells previously sensitized with potent anti-D serum were similarly sequestered in the spleen. With very potent sera (in terms of the P.V.P. test) the red cell disappearance rate was exponential. Less potent sera produced slower, diminishing rates of red cell destruction, in which various proportions of the sensitized red cells survived normally after an initial phase of rapid sequestration. That the red cell survival pattern was characteristic of the serum employed is evident in Figure 7, where repeated study of the survival of Cr^{51}-labelled cells sensitized *in vitro* provided reproducible results when the same serum and subject were used. As seen in Figure 7, this attempt at desensitization by repeated infusions of anti-D sensitized red cells did not alter the rate at which these cells were subsequently destroyed. These experiments with a homologous antibody failed to demonstrate the development of a protective "anti-antibody" mecha-

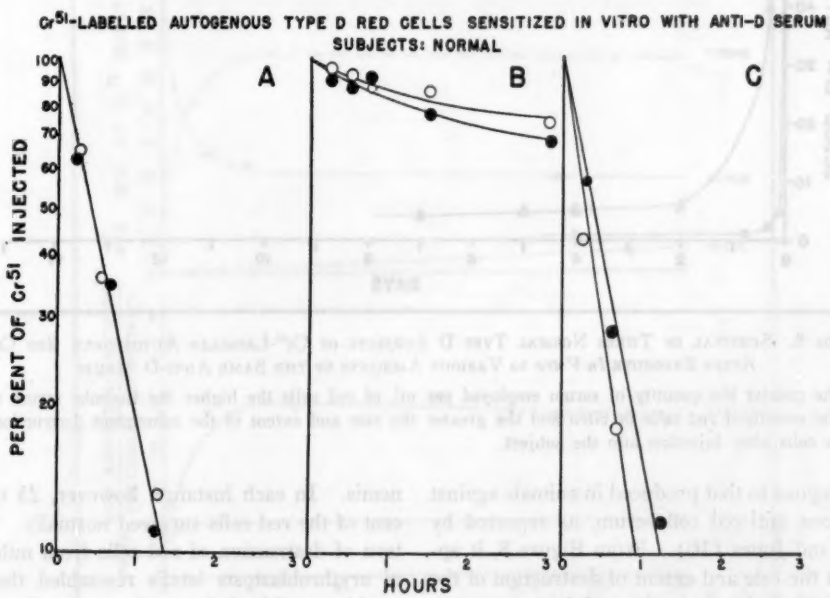

FIG. 7. NEGATIVE EFFECT UPON THEIR SUBSEQUENT RATE OF DESTRUCTION OF REPEATED INJECTIONS INTO NORMAL TYPE D SUBJECTS OF AUTOGENOUS RED CELLS PREVIOUSLY SENSITIZED *In Vitro* WITH INCOMPLETE ANTI-D SERUM

Samples of red cells of each of three subjects were sensitized *in vitro* with two volumes of the same antiserum and injected intravenously on five occasions over the course of three weeks. The red cells were labelled with Cr^{51} on the first (solid circles) and last (empty circles) occasions. The red cells of Subject A and of Subject C were sensitized with sera that produced strong agglutination of the cells in the "P.V.P. test." Their initial half-survival times were 27 and 20 minutes, respectively, and repeated injections of the sensitized red cells had no effect upon the subsequent survival of the sensitized autogenous cells. The serum employed in sensitizing the red cells of Subject B produced an equally intense Coombs reaction, but agglutinates of the sensitized cells that formed in the presence of P.V.P. slowly dissociated in saline. *In vivo* a relatively small portion of these cells was destroyed and splenic radioactivity rose slightly initially but not thereafter. Again repeated injections of the sensitized cells did not discernably affect their subsequent survival.

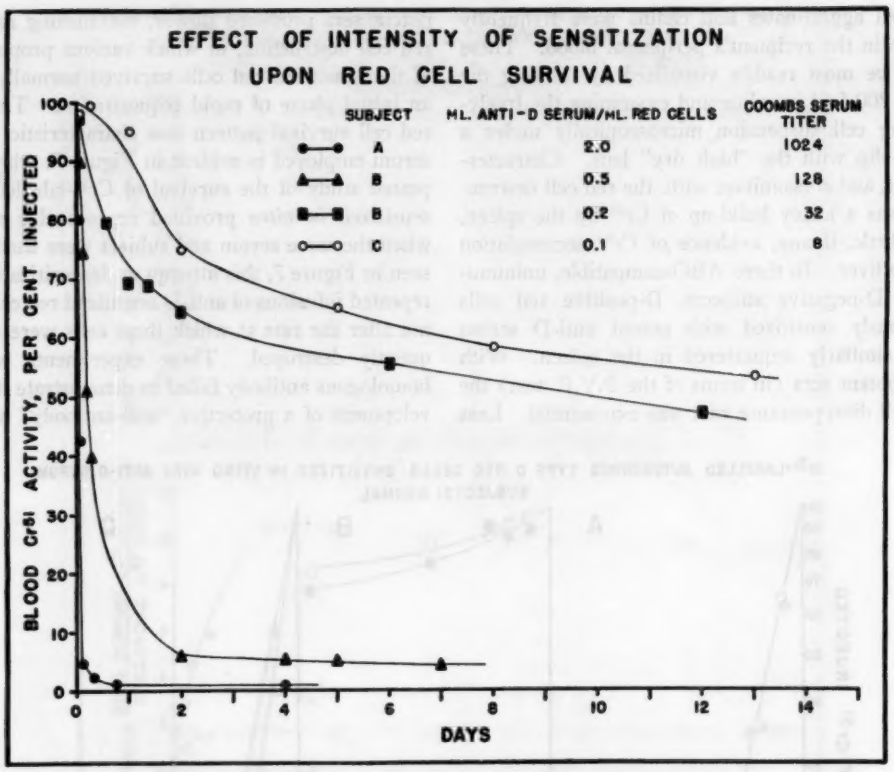

FIG. 8. SURVIVAL IN THREE NORMAL TYPE D SUBJECTS OF Cr^{51}-LABELLED AUTOGENOUS RED CELLS AFTER EXPOSURE *In Vitro* TO VARIOUS AMOUNTS OF THE SAME ANTI-D SERUM

The greater the quantity of serum employed per ml. of red cells the higher the Coombs serum titer of the sensitized red cells *in vitro* and the greater the rate and extent of the subsequent destruction of these cells after injection into the subject.

nism analogous to that produced in animals against heterologous anti-red cell serum, as reported by Samaille and Jones (16). From Figure 8, it appears that the rate and extent of destruction of the sensitized labelled cells in the recipient were proportional to the volume of anti-D serum relative to that of the red cells employed in the sensitization procedure.

Four studies were made of the destruction of Cr^{51}-labelled red cells from the cord blood of erythroblastotic infants after injection into ABO-compatible normal adult subjects. Figure 9 presents observations on the fate of such red cells from one of the two infants with severe erythroblastosis fetalis. In each observation there was rapid removal of the injected cells with evident splenic sequestration and only slight hemoglobinemia. In each instance, however, 25 to 30 per cent of the red cells survived normally. The pattern of destruction of red cells from milder cases of erythroblastosis fetalis resembled that of the weakly sensitized D-positive red cells shown in Figure 8. There was excellent agreement between the extent of destruction of erythroblastosis fetalis red cells transfused into adult recipients, the permanence on resuspension in saline of the red cell agglutinates produced by P.V.P., and the clinical severity of the hemolytic disease. On the other hand, the titer and intensity of the direct Coombs test did not show such a correlation.

The survival in a splenectomized subject of normal human red cells sensitized with incomplete anti-D serum was studied, employing in the sensitization procedure a serum known to cause seques-

tration of half of such sensitized cells by the spleen of a normal subject within 30 minutes. In the splenectomized subject the half-survival of these cells was over four and one-half hours, and their disappearance from the blood stream was attended by a pronounced uptake of Cr^{51} by the patient's liver (Figure 10).

In order to determine the effect *in vivo* of hyperglobulinemia upon the survival of sensitized red cells, a D-positive subject with multiple myeloma and very high serum globulin levels (over 12 Gm. per cent) was reinjected with her own red cells which had been labelled with Cr^{51} and sensitized with incomplete anti-D serum. Whereas in normal subjects the half-survival of red cells treated with this serum was about 30 minutes, in this patient the half-survival time was about five minutes and almost 90 per cent of the sensitized red cells

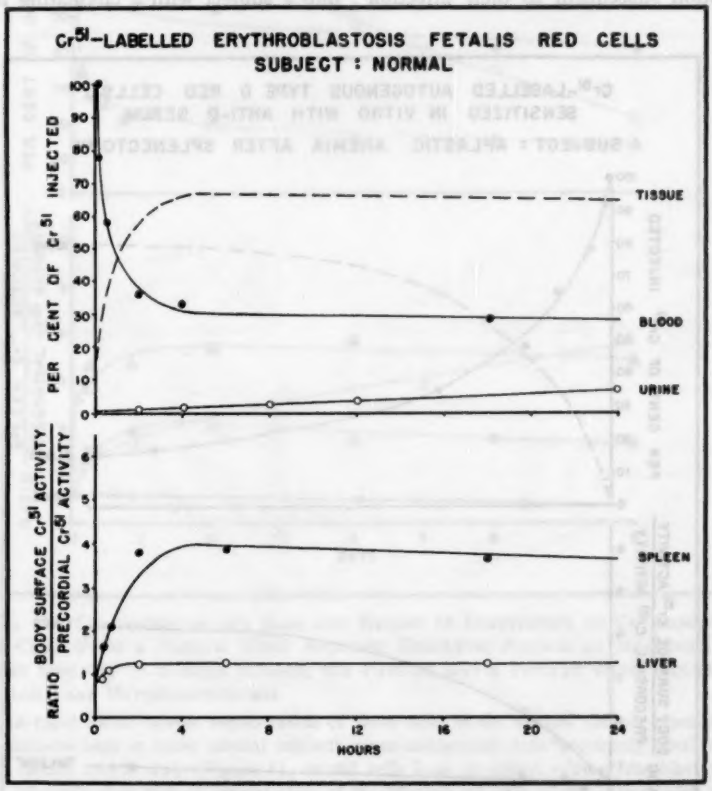

FIG. 9. SURVIVAL IN A NORMAL COMPATIBLE ADULT OF RED CELLS FROM THE CORD BLOOD OF AN INFANT WITH ERYTHROBLASTOSIS FETALIS

The red cells employed were removed prior to the first of three exchange transfusions required. They manifested agglutination in Coombs serum at a 1:2048 titer and were strongly agglutinated by P.V.P with no subsequent dispersion of these agglutinates on resuspension in saline during 24 hours at room temperature. Their osmotic fragility was normal and their mechanical fragility was slightly increased (10 per cent). As with red cells sensitized with potent anti-D serum *in vitro* (Figure 6), most of these red cells were rapidly sequestered by the spleen. However, almost 30 per cent of the red cells escaped destruction, as indicated. The infant's mother had a 1:64 titer in 25 per cent albumin of incomplete anti-D antibody, and after birth the child developed anemia and jaundice within a few hours and exhibited hepatosplenomegaly.

were sequestered within 10 minutes of injection. The pathologically sensitized red cells from a patient with a moderately severe acquired hemolytic anemia were simultaneously given to a normal subject and to the multiple myeloma patient (Figure 11). Here is shown the rapid sequestration of about 40 per cent of these red cells in the spleen of the normal subject, and the even more rapid and more complete sequestration by the impalpable spleen of the multiple myeloma patient.

The distribution subsequent to their injection into a dog of autogenous red cells sensitized *in vitro* to the Coombs test by their previous exposure to $CrCl_3$ is presented in Table I. Two hours after their injection, 37.1 per cent of these cells had disappeared from the circulation, and of these over two-thirds were removed by the spleen.

Destruction of sequestered red cells

Although the injection of small volumes of red cells (*i.e.*, containing about 1 Gm. of hemoglobin) into a subject with a circulating incomplete anti-

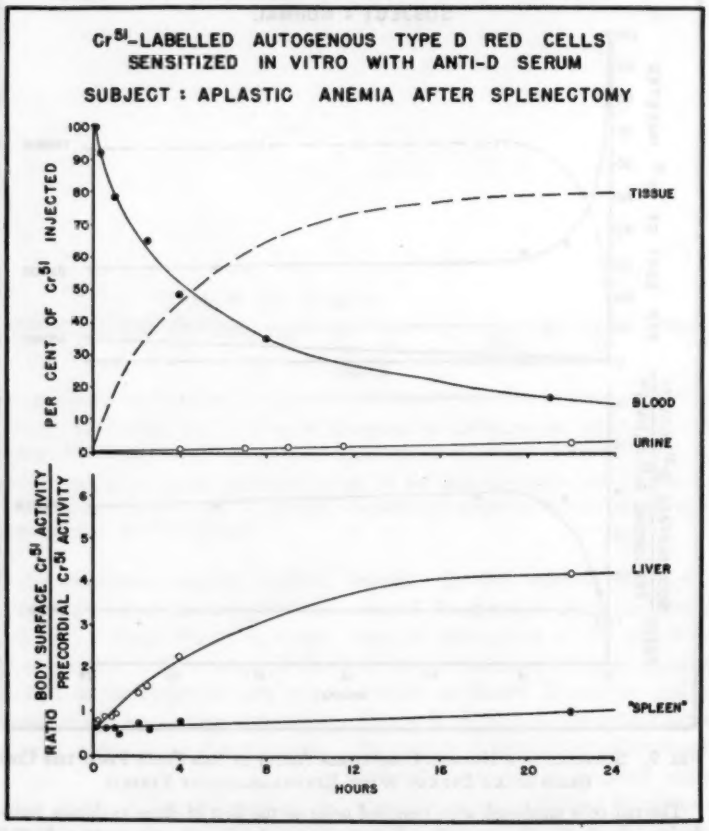

FIG. 10. DESTRUCTION IN A PREVIOUSLY SPLENECTOMIZED SUBJECT OF AUTOGENOUS TYPE D RED CELLS SENSITIZED *IN VITRO* WITH ANTI-D SERUM

Ten ml. of packed red cells from this adult subject with idiopathic aplastic anemia and a blood hemoglobin level of about 8 Gm. per cent were sensitized with the same incomplete anti-D serum that was used in the study depicted in Figure 6. In contrast to the half-survival of 30 minutes in that study, however, the half-survival of sensitized red cells in this splenectomized subject was prolonged to 4 hours and 40 minutes, and there was an appreciable accumulation of Cr^{51} in the subject's liver.

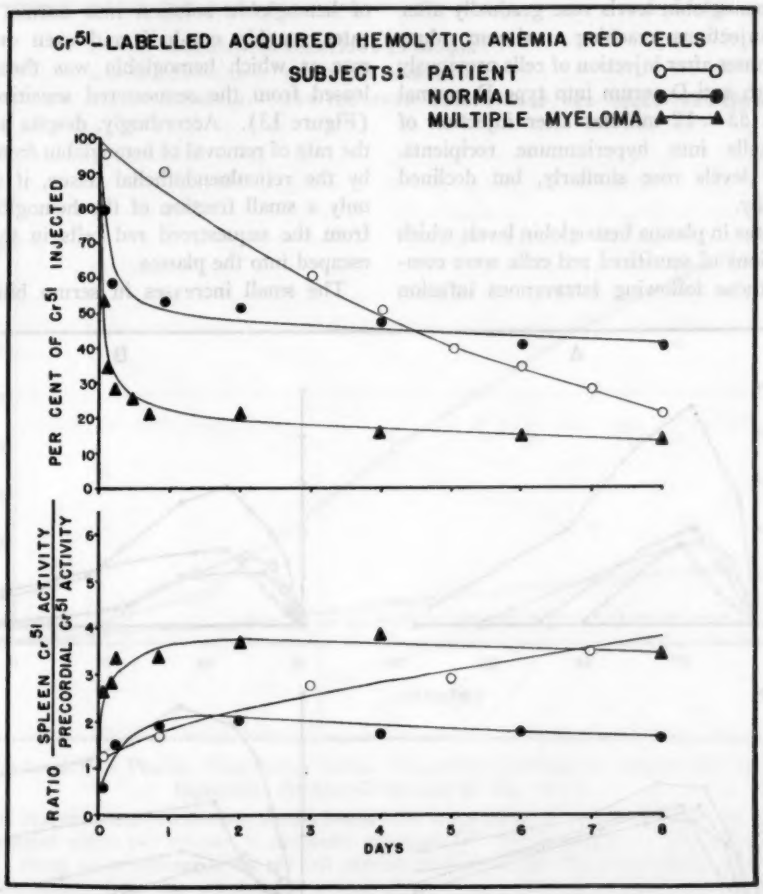

FIG. 11. COMPARISON OF THE RATE AND EXTENT OF DESTRUCTION OF Cr^{51}-LABELLED RED CELLS FROM A PATIENT WITH ACQUIRED HEMOLYTIC ANEMIA AS MEASURED IN THREE SUBJECTS: A NORMAL SUBJECT, THE PATIENT, AND A PATIENT WITH MULTIPLE MYELOMA AND HYPERGLOBULINEMIA

The rapid initial splenic sequestration of these cells in the normal subject resembles the patterns seen in other normal subjects given autogenous cells previously sensitized with certain anti-D sera (Figure 6), or red cells from an infant with erythroblastosis. The relatively slow rate of sequestration of the labelled red cells in the patient with acquired hemolytic anemia is believed to reflect the competition of the patient's circulating unlabelled cells for the splenic sequestering site. The red cells from the patient with acquired hemolytic anemia, as well as anti-D sensitized red cells (not shown), were sequestered even more rapidly and to a greater extent in the patient with multiple myeloma, whose hyperglobulinemic serum agglutinated the sensitized red cells *in vitro*. This patient's spleen was not palpable.

body, or the injection of similar volumes of previously sensitized red cells into normal subjects, was followed by little or no rise in plasma hemoglobin levels, the injection of larger volumes of cells produced detectable, albeit still low, levels of hemoglobin and of Cr^{51} in the plasma. In Figure 12 are depicted the individual levels of plasma hemoglobin and Cr^{51} following the injection of either type of red cell preparation containing 3.3 ± 0.5 Gm. of hemoglobin. As revealed in this fig-

ure plasma hemoglobin levels rose gradually after the red cell injections, reaching maximum values in 65 ± 5 minutes after injection of cells previously sensitized with anti-D serum into type D normal subjects and 53 ± 12 minutes after injection of D-positive cells into hyperimmune recipients. Plasma Cr^{51} levels rose similarly, but declined more gradually.

The increases in plasma hemoglobin levels which follow injections of sensitized red cells were compared with those following intravenous infusion of hemoglobin solution into normal subjects at rates roughly one-half, and even one-tenth, the rate at which hemoglobin was theoretically released from the sequestered sensitized red cells (Figure 13). Accordingly, despite allowance for the rate of removal of hemoglobin from the plasma by the reticuloendothelial tissue, it appears that only a small fraction of the hemoglobin released from the sequestered red cells in these subjects escaped into the plasma.

The small increases in serum bilirubin levels

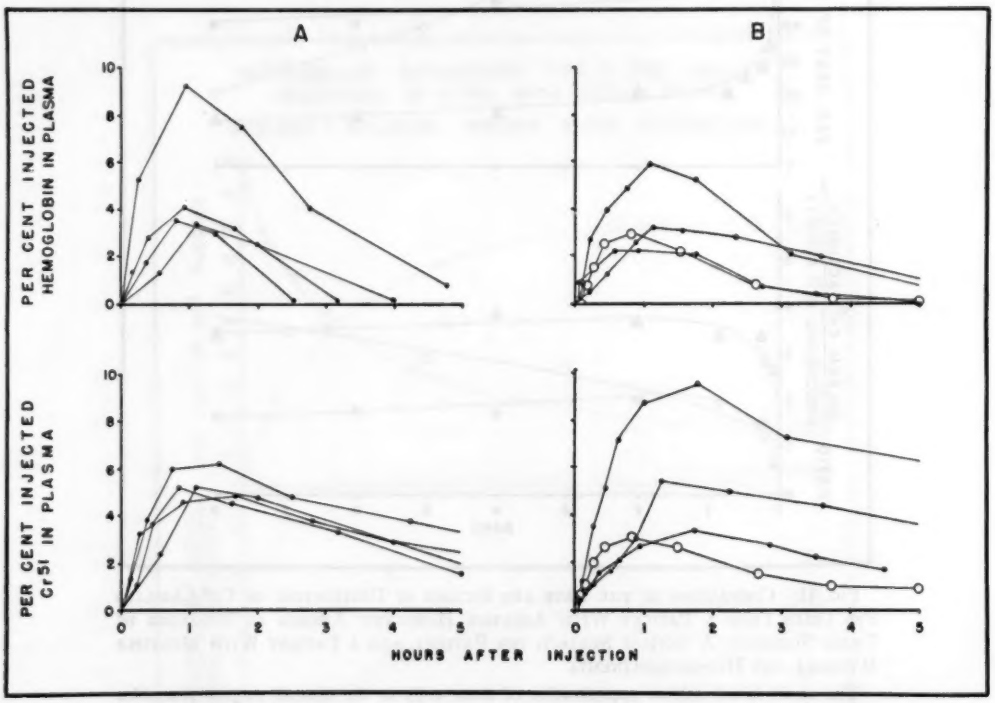

FIG. 12. PLASMA HEMOGLOBIN AND Cr^{51} LEVELS FOLLOWING THE INJECTION OF ANTI-D SENSITIZED RED CELLS

The studies portrayed under "A" were carried out on four D-negative otherwise normal subjects whose serum contained incomplete anti-D antibodies. Calculated total circulating plasma hemoglobin values in these subjects rose to between 3.3 and 8.6 per cent of the injected hemoglobin slightly less than one hour after injection. Similarly, total plasma Cr^{51} values reached peak levels of from 4.6 to 6.1 per cent of the injected radioactivity about one hour after injection. The studies under "B" were performed on four normal subjects who received red cells sensitized *in vitro* with potent incomplete anti-D antibodies. In the three type D subjects (solid circles) maximum values of from 2.1 to 5.8 per cent of the hemoglobin injected as sensitized autogenous cells appeared in the plasma a little more than an hour later; and the plasma Cr^{51} levels reached peak values of from 3.4 to 9.5 per cent of the injected dose about one and one-half hours after injection. The fourth subject (open circles), who was D-negative, showed similar findings but at a somewhat accelerated pace resembling that of the sensitized subjects under "A." The hemoglobin content of the injected cell suspensions ranged from 2.8 to 3.8 Gm. Thus, in both groups of subjects, only a small fraction of the injected hemoglobin appeared in the plasma. The regularly occurring gradual rises in plasma hemoglobin to maximum levels about one hour after the red cell injections suggest that the period of maximum hemolysis came within that hour.

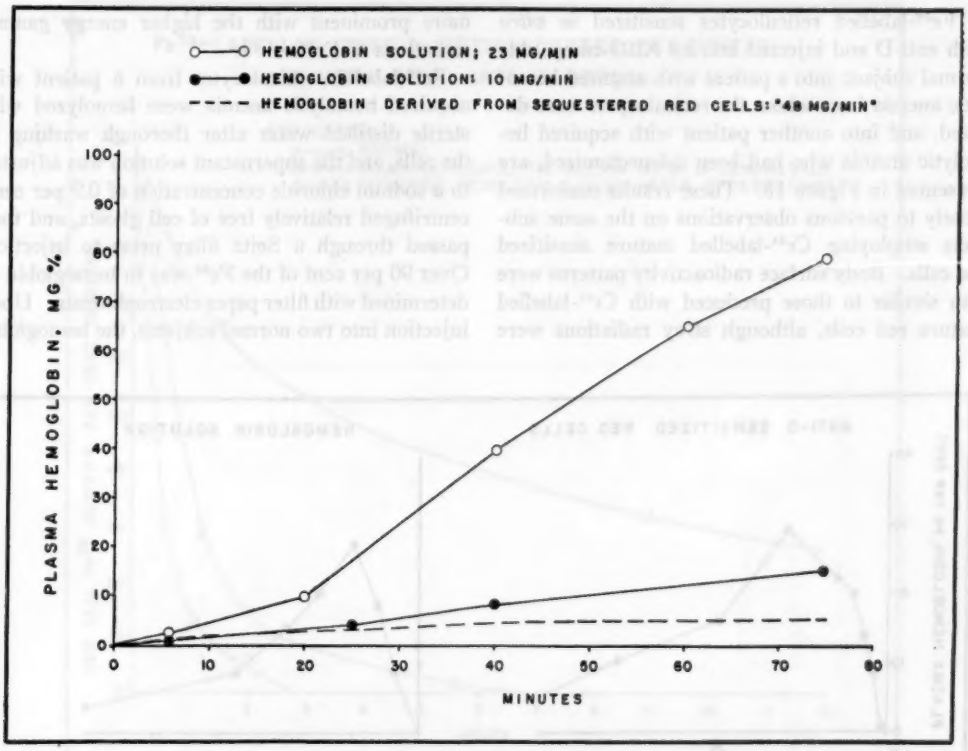

FIG. 13. COMPARISON OF PLASMA HEMOGLOBIN LEVELS FOLLOWING INFUSIONS OF HEMOGLOBIN SOLUTION AND INJECTIONS OF ANTI-D SENSITIZED RED CELLS

The dotted line represents the average plasma hemoglobin levels of seven normal subjects who received injections completed within two minutes of previously sensitized red cells containing an average of 3.1 Gm. of hemoglobin. Based on an average of the red cell survival studies, the quantity of hemoglobin released if these cells were immediately lysed was calculated to average about 60 mg. per minute during the first 26 minutes and about 36 mg. per minute during the next 34 minutes, with an overall average release of hemoglobin during the first hour of about 48 mg. per minute. Despite this potential rate of delivery of hemoglobin into the plasma, the maximum level reached at the end of one hour after injection of these cells averaged only about 5 mg. per cent above the base line. In contrast a steady infusion into a normal subject of hemoglobin solution at the rate of only 10 mg. per minute produced a plasma hemoglobin level at one hour of 12.5 mg. per cent; and the infusion of 23 mg. of hemoglobin per minute produced a plasma level of about 65 mg. per cent at one hour.

after the injection of anti-D sensitized red cells containing about 3 Gm. of hemoglobin into normal subjects were of doubtful significance. To observe better the change in serum bilirubin levels one normal subject was injected with a quantity of anti-D sensitized autogenous red cells containing 6.9 Gm. of hemoglobin. As revealed in the left portion of Figure 14, this led to a higher, more protracted rise in plasma hemoglobin levels than was seen after injection of smaller quantities of sensitized cells. An increase in the total serum bilirubin level first appeared about one hour after injection and rose to a maximum in about five hours, while a rise in the level of prompt direct-acting bilirubin was first evident between two and three hours, and also reached a maximum about five hours after injection. For comparison, hemoglobinemia of a similar magnitude was produced by the slow intravenous infusion of hemoglobin solution; this produced a comparatively small rise in serum bilirubin levels.

Further evidence of the speed of breakdown of sequestered red cells was sought by the use of Fe^{59}-labelled reticulocytes. The rates of sequestration

of Fe^{59}-labelled reticulocytes sensitized *in vitro* with anti-D and injected into an ABO-compatible normal subject, into a patient with acquired hemolytic anemia from whom the reticulocytes were derived, and into another patient with acquired hemolytic anemia who had been splenectomized, are presented in Figure 15. These results conformed closely to previous observations on the same subjects employing Cr^{51}-labelled mature sensitized red cells. Body surface radioactivity patterns were also similar to those produced with Cr^{51}-labelled mature red cells, although stray radiations were more prominent with the higher energy gamma radiations of Fe^{59}.

Fe^{59}-labelled reticulocytes from a patient with acquired hemolytic anemia were hemolyzed with sterile distilled water after thorough washing of the cells, and the supernatant solution was adjusted to a sodium chloride concentration of 0.9 per cent, centrifuged relatively free of cell ghosts, and then passed through a Seitz filter prior to injection. Over 90 per cent of the Fe^{59} was in hemoglobin as determined with filter paper electrophoresis. Upon injection into two normal subjects, the hemoglobin

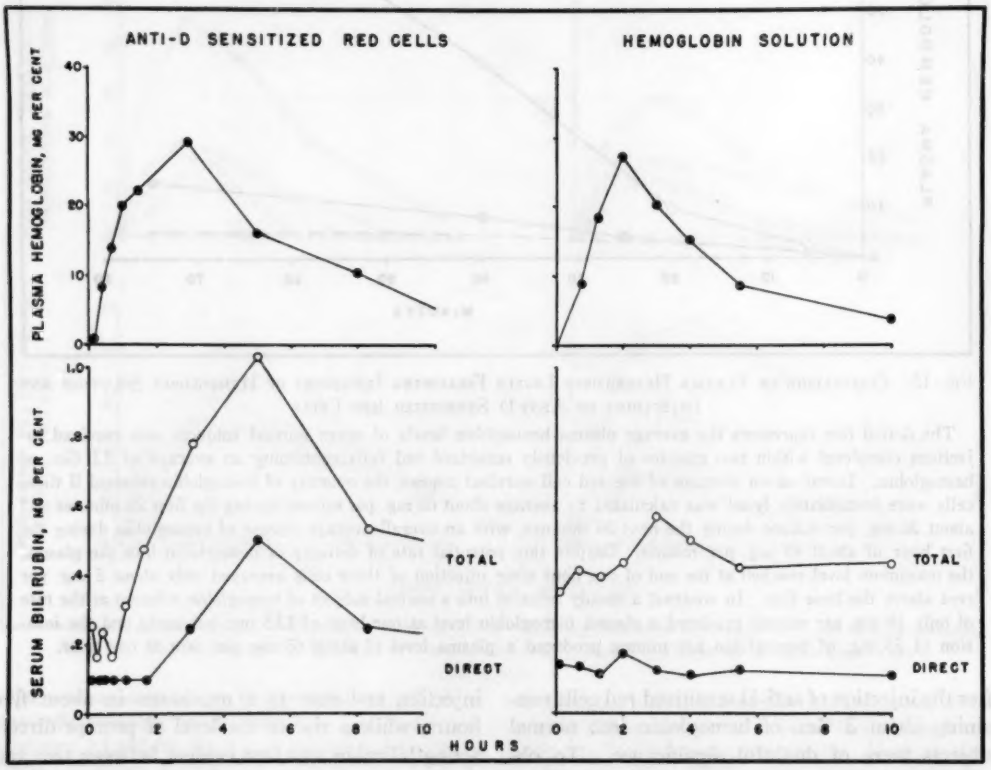

FIG. 14. PLASMA HEMOGLOBIN AND SERUM BILIRUBIN LEVELS IN NORMAL SUBJECTS INJECTED EITHER WITH ANTI-D SENSITIZED AUTOGENOUS RED CELLS OR WITH HEMOGLOBIN SOLUTION

The injection of sensitized red cells (left above) containing 6.9 Gm. of hemoglobin led to an appreciably higher and more prolonged rise in plasma hemoglobin levels than was the case with smaller injections (Figures 4, 5, 6 and 11), although the rate of red cell sequestration was similar. Beginning an hour after the injection a rise in total serum bilirubin levels appeared, reaching a maximum in five hours. The initial rise consisted entirely of "indirect" reacting bilirubin; not until over two hours after injection did the prompt "direct" reacting bilirubin level rise. A similar degree of hemoglobinemia produced by slow infusion of hemoglobin solution (right above) caused only a relatively small and questionable increase in bilirubin levels. These data are interpreted to mean that most or all of the sequestered sensitized red cells were hemolyzed *in situ* and that this occurred within a short time of their sequestration.

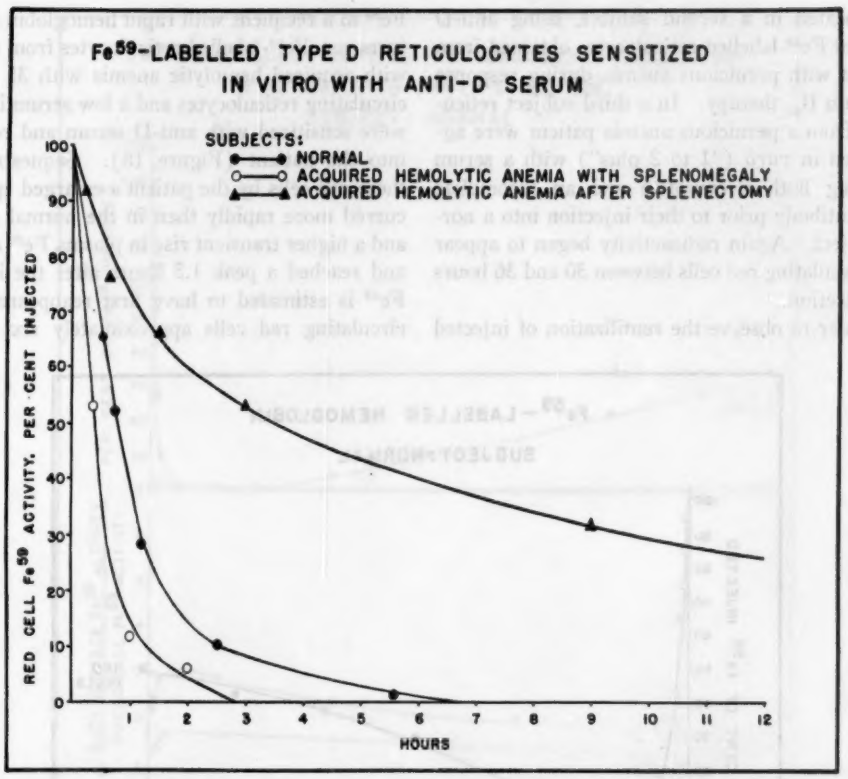

FIG. 15. DESTRUCTION OF Fe^{59}-LABELLED RETICULOCYTES PREVIOUSLY SENSITIZED *IN VITRO* WITH INCOMPLETE ANTI-D SERUM AND THEN INJECTED INTO THREE DIFFERENT RECIPIENTS: A NORMAL SUBJECT, A PATIENT WITH AN ACTIVE ACQUIRED HEMOLYTIC ANEMIA AND SPLENOMEGALY, AND A PATIENT CONVALESCING FROM AN ACQUIRED HEMOLYTIC ANEMIA SEVERAL MONTHS AFTER SPLENECTOMY

The rates of destruction of these sensitized cells closely resemble those determined in the same subjects with Cr^{51}-labelled mature red cells. In the first two subjects prominent levels of Fe^{59} activity appeared in the spleen, whereas in the splenectomized subject a comparatively high hepatic radioactivity developed (not shown) reciprocally with the fall in circulating red cell Fe^{59} activity.

(benzidine) half-disappearance rates from the plasma were 2.2 and 2.4 hours and those of the Fe^{59} were 2.7 and 3.0 hours, respectively. A high relative hepatic radioactivity attended the removal of Fe^{59} from the blood. Radioactivity first appeared in these subjects' red cells about 18 hours after injection, and increased steadily thereafter while hepatic radioactivity gradually diminished (Figure 16).

The effect of the injection of anti-D sensitized, Fe^{59}-labelled reticulocytes from a patient with acquired hemolytic anemia into a normal subject is shown in Figure 17. Here the Fe^{59} disappeared rapidly from the circulating red cells with a half-survival time of 40 minutes. As in the Cr^{51} studies, plasma hemoglobin levels reached a maximum about one hour after injection. The plasma Fe^{59} peak was not reached until two and one-half hours after injection. Concurrent with the red cell Fe^{59} disappearance, a high relative splenic radioactivity developed. Thereafter, red cell radioactivity remained low until from 30 to 36 hours after injection, when radioactivity began to accumulate progressively in the circulating red cells while splenic radioactivity steadily diminished. An almost identical time relationship occurred when this study

was repeated in a second subject, using anti-D sensitized Fe^{59}-labelled reticulocytes obtained from a patient with pernicious anemia during response to vitamin B_{12} therapy. In a third subject reticulocytes from a pernicious anemia patient were agglutinated *in vitro* ("1 to 2 plus") with a serum containing both a complete and an incomplete anti-C antibody prior to their injection into a normal subject. Again radioactivity began to appear in the circulating red cells between 30 and 36 hours after injection.

In order to observe the reutilization of injected Fe^{59} in a recipient with rapid hemoglobin and iron turnover, Fe^{59}-labelled reticulocytes from a patient with acquired hemolytic anemia with 35 per cent circulating reticulocytes and a low serum iron level were sensitized with anti-D serum and reinjected into the patient (Figure 18). Sequestration of these red cells by the patient's enlarged spleen occurred more rapidly than in the normal subjects, and a higher transient rise in plasma Fe^{59} appeared and reached a peak 1.5 hours after the injection. Fe^{59} is estimated to have first reappeared in the circulating red cells approximately six or eight

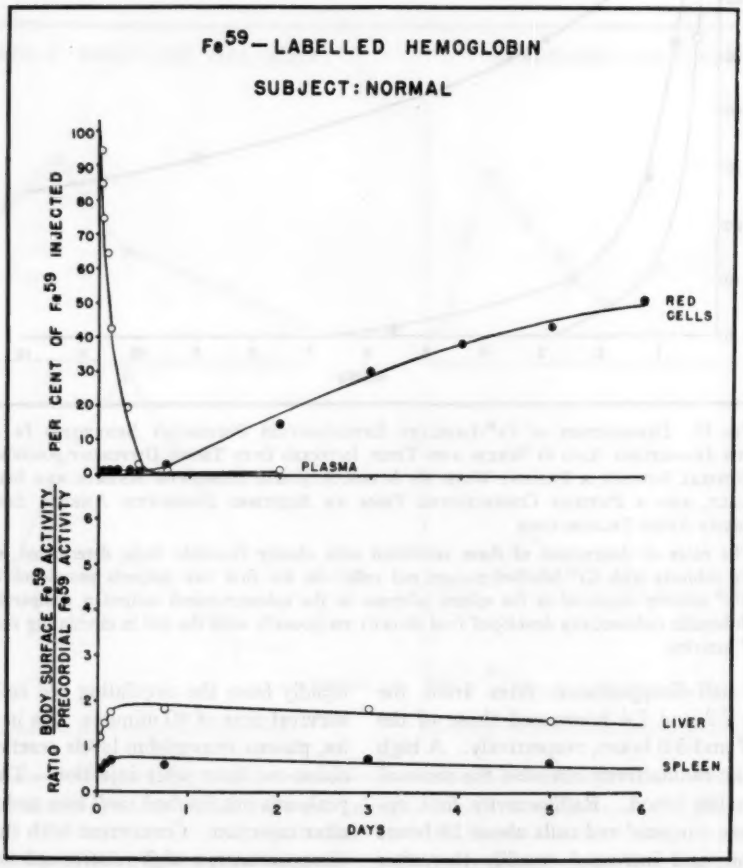

FIG. 16. REUTILIZATION IN A NORMAL SUBJECT OF Fe^{59} INJECTED AS Fe^{59}-LABELLED HEMOGLOBIN

After injection of the radioactive hemoglobin solution, half of the Fe^{59} activity disappeared from the plasma within 2.6 hours, and all was gone in 12 hours. Meanwhile, radioactivity appeared and reached its maximum value in the subject's liver. Radioactivity was first detectable in small amounts in the subject's red cells 18 hours later, and within 10 days had accumulated to a total of 60 per cent of the injected dose.

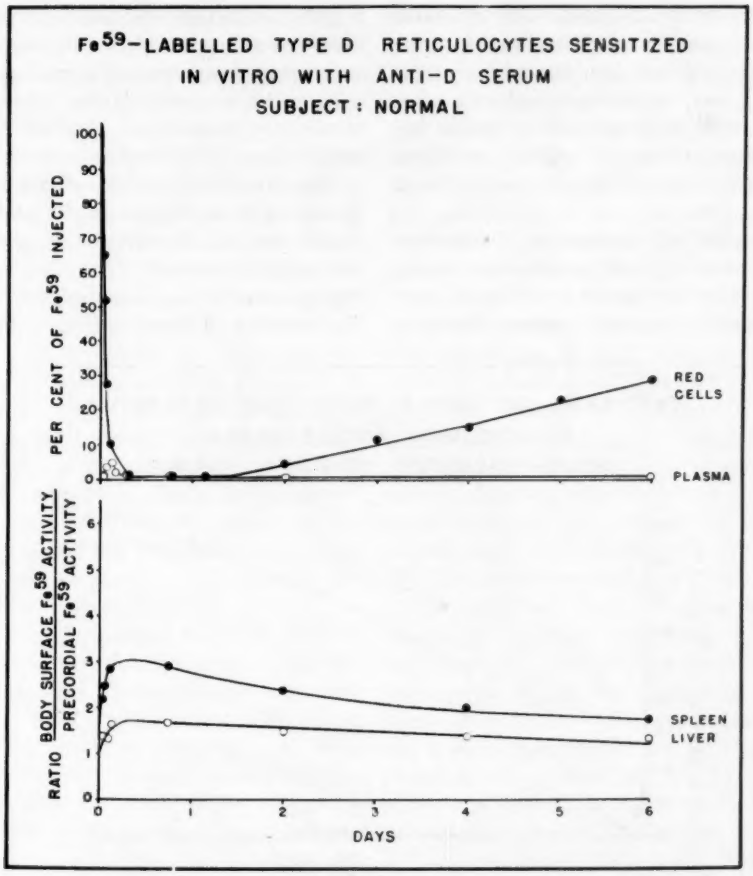

FIG. 17. REUTILIZATION IN A NORMAL SUBJECT OF Fe^{59} INJECTED AS Fe^{59}-LABELLED ANTI-D SENSITIZED RETICULOCYTES

Although the rate of disappearance of Fe^{59} from the blood was more rapid (half-disappearance time: 40 minutes) than following the injection of Fe^{59}-labelled hemoglobin solution (Figure 13), reappearance of Fe^{59} in the circulating red cells was first detected at 48 hours instead of at 18 and, as estimated by extrapolation, first appeared in the red cells 34 to 36 hours after injection. The prompt and relatively high splenic radioactivity declined more rapidly than after comparable experiments employing Cr^{51}-labelled red cells.

hours after injection and increased rapidly to very high levels, along with a moderate fall in splenic radioactivity. A later gradual rise in radioactivity over the spleen presumably reflected sequestration of newly produced red cells as a manifestation of the disease process.

DISCUSSION

The injection into a normal subject of Cr^{51}-labelled red cells previously sensitized with potent incomplete anti-D antibodies leads to the rapid almost total filtration of these cells by the normal spleen, as indicated by a striking rise in splenic radioactivity. Although not agglutinated by the sensitizing serum or by the recipient's plasma, half of these cells are sequestered within about 25 minutes. During this period small numbers of agglutinated red cells are visible in the recipient's blood, but hemoglobin appears in the plasma only in relatively small amounts reaching a maximum level 60

to 70 minutes after the injection. That the same sequence ensues after the injection of anti-D sensitized D-positive red cells into D-negative recipients, indicates that the normal mechanisms for filtering these cells do not require a specific immune interaction between cell "coating" and filter. Rather the spleen appears to act passively as a highly efficient filter.

That the quality and the quantity of antiserum used in the passive red cell sensitization experiments are critical to the manner in which the sensitized red cells are subsequently destroyed *in vitro*, is illustrated in Figures 7 and 8. Presumably such factors were responsible for the normal survival, after initial destruction of a small portion of the cells, of the remainder of the red cells sensitized *in vitro* by Mollison and Paterson (50). Loutit and Mollison (51) found that red cell agglutinates produced *in vitro* by anti-A serum appeared largely to disperse in the circulation of type A subjects, in that the survival of such pre-agglutinated red cells was relatively normal. The observations on the first of our subjects so studied, however, indicate that retention of the red cell agglutinates may oc-

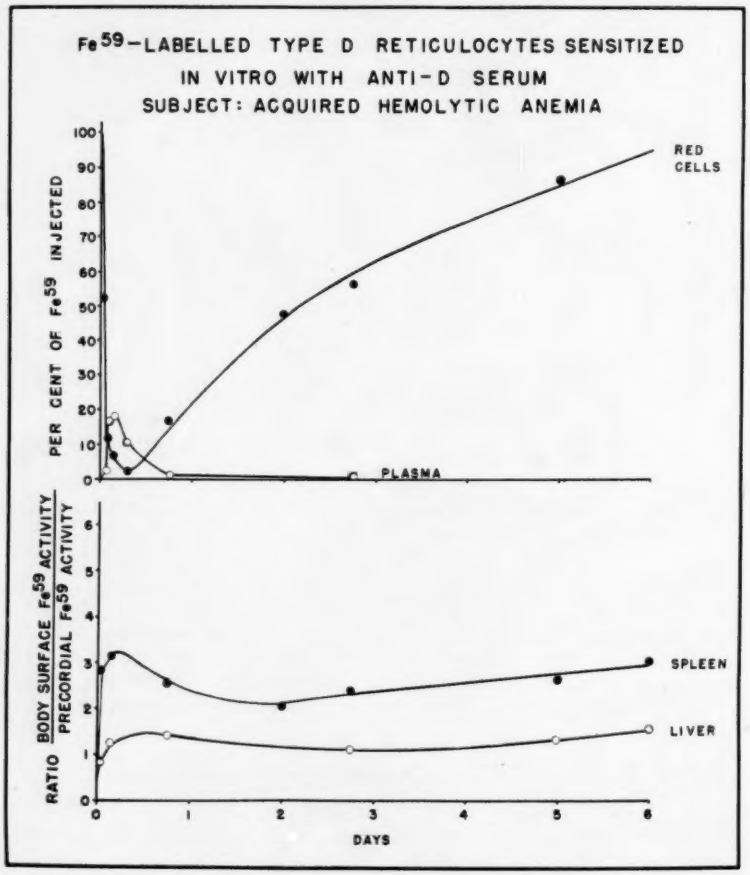

FIG. 18. REUTILIZATION IN A SUBJECT WITH ACQUIRED HEMOLYTIC ANEMIA AND IRON DEFICIENCY OF Fe^{59} INJECTED AS Fe^{59}-LABELLED ANTI-D SENSITIZED RETICULOCYTES

In contrast to their fate in the normal subject (Figure 14) these reticulocytes were sequestered more rapidly, plasma Fe^{59} levels reached higher values, and Fe^{59} reappeared more quickly in the circulating red cells (within six or eight hours of injection) in association with a more rapidly-diminishing splenic radioactivity.

cur before such dispersion has come about and that this retention occurs to a large extent in the liver. That this temporary sequestration may persist for an hour or so with only a fraction of the retained cells becoming hemolyzed is in contrast with the permanent sequestration and the apparent rapid hemolysis ensuing in the experiments involving anti-D and anti-C. It has been shown that when red cells are sensitized with various anti-D sera and are then agglutinated by substances which cause rouleaux, the permanence of these agglutinates when they are subsequently suspended in saline varies with the particular antiserum employed; these differences in the agglutinates of red cells sensitized with various incomplete anti-D sera are not due to simple elution of the antibodies but reflect qualitative differences in the "stickiness" induced by the antibodies (30). In the anti-D experiments described above, the degree of destruction of sensitized red cells *in vivo* correlated well with the persistence of P.V.P.-induced agglutinates of these cells *in vitro*.

Type D Cr^{51}-labelled normal red cells, given to D-negative subjects with circulating incomplete anti-D antibodies, are removed somewhat more rapidly than in the experiments described above on normal D-positive subjects injected with D-sensitized red cells. Sequestration of the injected cells was less prominent in the spleens of sensitized subjects, and a considerable hepatic uptake of radioactive cells was also demonstrable, particularly when sequestration was most rapid. In these subjects, also, plasma levels of hemoglobin and of Cr^{51} rose slowly after injection to low maximum levels about 50 to 60 minutes after injection.

In D-negative subjects with both complete and incomplete anti-D or anti-C antibodies, red cell sequestration occurred even more rapidly, and hepatic sequestration was still more prominent, in some cases exceeding that of the spleen. The difference in body distribution of Cr^{51} in hyperimmunized subjects from that of normal subjects given previously-sensitized red cells might reflect simply a difference in the quantity of antibody. However, the fact that a comparatively large hepatic uptake may occur in hyperimmunized subjects even when the disappearance rate of injected red cells is similar to that of normal subjects receiving presensitized cells is more suggestive of qualitative rather than a quantitative difference in the mechanisms of hemolysis. Thus, the more striking hepatic sequestration of type D cells in subjects with circulating incomplete anti-D antibody suggests that the filtering mechanism here may be specifically sensitized to the red cell antigen, in addition to operating simply as a passive filter. Visible evidence of such an interaction between type D red cells and the granulocytes of sensitized subjects has been noted recently by one of the authors (49). Indeed, clumping *in vitro* of the red cells of patients with acquired hemolytic anemia around the patients' leukocytes was observed as an early stage of erythrophagocytosis by Zinkham and Diamond (52). Swisher (53) has reported the appearance of mixed agglutinates of canine red cells, leukocytes and platelets when the red cells were exposed to canine anti-A. Further work along this line will be required to explain the difference in the pattern of sequestration of sensitized red cells by normal as compared to hyperimmune recipients.

The brisk and peculiar fashion in which the normal spleen filters sufficiently sensitized red cells from the circulation without intravascular hemolysis suggests that a physical or physicochemical mechanism involving the surfaces of the red cells is operative, rather than one involving metabolic changes within the red cells. The spleen's proficiency as a discriminating filter for spherocytes (54–57) and certain other particles (58) is well established and can be explained on purely mechanical grounds. Since the size, shape and viscosity of red cells are unaltered by sensitization with incomplete antibodies, an explanation must be sought in their tendency either to adhere to one another or to other cells. The belief that red cell agglutination is an intermediate step in the destruction of sensitized cells became tenable with the demonstration of a "conglutinating" action of normal plasma (28) and more recently by the finding that all rouleaux-producing substances, including fibrinogen and the other plasma globulins, categorically are active in this respect (30). Although the agglutination-enhancing effect of most of the tube tests employing serum as a diluent and of the slide test for incomplete antibodies (59) depends upon some degree of evaporation and the formation of surface layers of protein when normal serum is used, undehydrated plasma from normal persons often produces a slight partial ag-

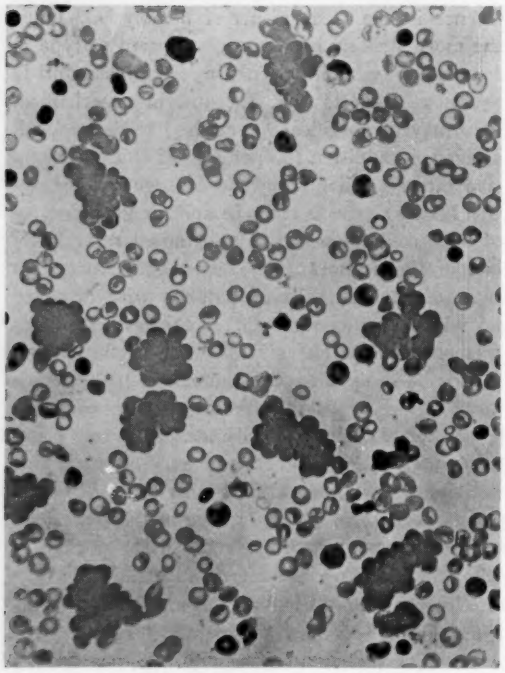

FIG. 19. RED CELL AGGLUTINATES IN THE SPLENIC PULP BLOOD OF A PATIENT WITH ACQUIRED HEMOLYTIC ANEMIA AND NO PERIPHERAL AUTOAGGLUTINATION

This smear was prepared from blood escaping from the freshly-incised surface of the patient's spleen shortly after its surgical excision. This blood revealed an icterus index of 50, a 2 to 3 plus Coombs test, a 4 plus P.V.P. test, and 2 plus agglutination in saline, while the peripheral blood drawn at the same time showed an icterus index of 12, a 2 plus Coombs test, a 4 plus P.V.P. test and no agglutination in saline. Incubation of the splenic pulp blood plasma with normal red cells produced evidence of red cell sensitization but not of agglutination. In spite of the impression that the agglutinated cells appear dense and spheroidal, the osmotic fragility of the splenic pulp red cells was only slightly greater than that of peripheral venous blood red cells (50 per cent hemolysis at 0.55 Gm. per cent NaCl compared to 0.51 Gm. per cent, respectively).

glutination of red cells sensitized with incomplete antibodies, and in persons with high sedimentation rates this effect may be pronounced (30, 60). The observations reported above on the accelerated sequestration of sensitized red cells injected into a subject with hyperglobulinemia support the contention that the concentrations *in vivo* of those substances which cause rouleaux *in vitro* (notably fibrinogen and other globulins) are critical to the sequestering mechanism.

In three cases of acquired hemolytic anemia not included in the studies reported above, comparisons were made between the survival patterns of the patients' Cr^{51}-labelled red cells in the patients themselves and in normal subjects. The results closely resembled those depicted for the "patient" and the "normal" subject in Figure 11. Analogous observations have also been made by the authors upon the survival of red cells from a patient with hereditary spherocytosis in a normal subject and in the patient himself. Thus, small volumes of red cells from patients with hemolytic anemias were consistently destroyed with greater initial rapidity when injected into compatible normal subjects than when reinjected into the patients themselves. This probably reflects the smaller volume of abnormal red cells relative to the volume of filtering tissue in the normal recipient and thus indicates that the patients' red cells competitively occupy the filter receiving the labelled cells. Presumably it is on this basis that the destruction of small volumes of anti-D sensitized red cells described in this report proceeds so much more rapidly in normal subjects than in the corresponding clinical state of erythroblastosis fetalis. This preferential filtration of small volumes of anomalous red cells undoubtedly favors the detection of red cell incompatibility by trial transfusions of very small quantities of isotope-labelled red cells into patients suspected of being immunized to the red cells in question, as reported by Mollison and Cutbush (61).

On the other hand, if one assumes that the rate of filtration of abnormal red cells from the circulation in hemolytic anemias is limited by the available volume of sequestering tissue, then necessarily the disappearance rate of a given volume of patients' labelled abnormal red cells will be inversely proportional to the total volume (labelled and unlabelled) of abnormal red cells in the circulation. In this sense study of the survival of small volumes of labelled, pathological red cells reinjected into an afflicted patient is a tracer study of the turnover rate of the patient's entire red cell mass and can be analyzed accordingly; however, study of the survival of red cells taken from a patient with hemolytic anemia and injected into a normal subject is in no sense a tracer study and may provide mis-

leading evidence for too rapid a rate of red cell turnover in the patient.

Direct evidence of the role of red cell agglutination in the destruction of sensitized cells was the finding that circulating agglutinates appeared in the peripheral blood of normal subjects receiving previously sensitized but unagglutinated red cells, providing the rate of removal was not so rapid as apparently to preclude such an observation. Blood obtained at operation from the freshly-incised spleen of a patient with acquired hemolytic anemia, a positive Coombs test in the absence of spontaneous autoagglutination, and a high splenic uptake of Cr^{51}-labelled autogenous red cells, contained, in contrast to the peripheral blood, numerous red cell agglutinates (Figure 19). This finding further supports the intermediate role of agglutination, although conceivably the spleen may contain levels of an agglutinating antibody not apparent in the peripheral blood. Studies in this laboratory of the spleen blood of dogs anesthetized with Nembutal® indicate that not only was there a marked concentration of red cells, as originally observed by Barcroft (62, 63), but that the plasma protein concentrations were also increased in the spleens of four of five dogs studied (Table II). Since splenic hematocrit levels in these dogs reached as high as 97 per cent,[10] it is apparent that even the minor increases generally observed in the osmotic fragility (and thus in the water content) of "splenic" red cells would of necessity dehydrate the suspending plasma. Any increase in the concentration of plasma globulins (including fibrinogen) would, as stated above, induce or intensify red cell agglutination. However, whether or not the greater tendency of sensitized red cells to agglutinate in the blood stream of normal subjects than in their whole blood *in vitro* is an expression of local hyperproteinemia is not fully clarified.

Once sequestration of sensitized red cells in the spleen has occurred by virtue of their agglutinability, it appears that their actual destruction follows within a matter of minutes. This was apparent in several ways. Following the injection of sufficient volumes of D-positive red cells "coated" with incomplete anti-D serum into normal subjects, or of D-positive red cells into hyperimmunized D-negative subjects, plasma hemoglobin levels began to increase within a few minutes, and generally reached maximum levels approximately one hour later (Figure 12). The average half-maximum plasma hemoglobin level followed the average red cell half-survival time by less than 10 minutes, and the rises in plasma hemoglobin levels were roughly indirectly proportional to the decline in red cell Cr^{51} activity. The patterns of the plasma Cr^{51} levels resembled those of the plasma hemoglobin values except for their slower decline, a difference that has been observed with injections of Cr^{51}-labelled hemoglobin and that apparently derives from a gradual elution of Cr^{51} from hemoglobin *in vivo* (33). These observations on plasma hemoglobin and Cr^{51} levels are most consistent with the interpretation that a single hemolytic process exists and that only a small fraction of the released hemoglobin is returned to the circulation. The alternative explanation, that only a small fraction of the injected red cells is hemolyzed by one mechanism intravascularly while the majority of the cells are being sequestered (but not hemolyzed by another mechanism until later), is somewhat objectionable in that it requires the intravascular hemolytic processes affecting the lesser fraction of cells to be almost synchronous with the sequestering process affecting the greater fraction.

Further evidence that most or all of the sensitized red cells were hemolyzed soon after their

TABLE II

Comparison of the red cell and protein concentrations of the large vessel and splenic blood of five male dogs under Nembutal® anesthesia

	Hematocrit, vol. %		Plasma total protein, Gm. %			
Dog	Aorta	Spleen	Vena cava	Vena porta	Aorta	Spleen*
S-1	46.0	90.0	5.47	5.59	4.79	7.38
S-2	43.0	83.0	6.83	7.54	7.55	6.21
S-3	46.0	93.0	6.50	6.03	6.47	9.53
S-4	42.5	80.2	5.50	6.00	5.24	7.06
S-5	53.6	97.0	5.53	5.20	5.70	7.13
Average	46.2	88.6	5.97	6.07	5.95	7.46

* The plasma hemoglobin level of the spleen blood ranged from 0.12 to 0.65 (average, 0.31) Gm. per cent. At all other sites sampled the plasma hemoglobin levels were 0.01 Gm. per cent or less.

[10] Although many relaxing and anesthetic agents profoundly affect the hemodynamics of dog and cat spleens, rendering it difficult to translate such extreme hemoconcentration into human physiology, indirect measurements indicate that hemoconcentration exists in patients with splenomegaly unexposed to such medications (64).

sequestration emerged from studies of a subject injected with a relatively large quantity of anti-D sensitized autogenous red cells (Figure 14). In this subject the serum level of indirect-reacting bilirubin began to rise within an hour of injection of the sensitized red cells, and this rise reflected the breakdown of a much larger amount of hemoglobin than had appeared in the plasma. A similar disparity between the effects of intravenous injection of hemoglobin solutions and of plasma containing high titers of incompatible isoagglutinins on the plasma hemoglobin and bilirubin levels in normal subjects was observed by Ebert and Emerson (25). These workers stressed the relatively greater increase in serum bilirubin levels following the action *in vivo* of incompatible isoagglutinins, and reported that maximum increases in bilirubin levels occurred between two and six hours of the incompatible plasma injection. The time sequence of the bilirubin rise following injection of sensitized red cells depicted in Figure 14 may be compared with that observed by Duesberg (65) following direct intravenous infusions of large amounts of hemoglobin solution. In his observations serum bilirubin levels began to increase within one hour and reached maximum values in three to four hours.

In discriminating between "intravascular" and "extravascular" hemolysis it is generally difficult to be certain whether the assumed criteria represen[t] qualitative or quantitative differences. The data cited in Figure 13 stress a prominent qualitative difference between lysis of red cells in the general circulation and that in sequestering organs. Based on comparisons of these data and the assumption that the rise in plasma hemoglobin levels after the injection of sensitized red cells reflects a single lytic process affecting all the sequestered red cells, one may estimate that about 90 per cent of the derived hemoglobin is retained within the spleen and other sequestering sites and is largely catabolized *in situ*. This finding is consistent with our observations on a patient with acquired hemolytic anemia, in whom the bilirubin level of the splenic vein blood was almost five times as high as that of the peripheral blood, although the plasma hemoglobin level of the splenic vein blood was only slightly higher than that of the peripheral venous blood.

The fact that the Fe^{59} of sequestered reticulocytes reappeared in newly-formed red cells within as short a time as six or eight hours after its injection, indicates that hemolysis in the spleen, catabolism of the released heme, and incorporation of the released iron by immature red cells were all accomplished within this span of time. In addition, corroborating previous studies by Ross (66), these observations indicate that the iron of injected hemoglobin solutions is available for erythropoiesis at approximately the same rate as iron attached to the plasma iron-binding protein. Thus, the Fe^{59} disappearance and reutilization curves of Figure 16 resemble closely the composite values for injected plasma-bound radioiron for normal subjects compiled by Finch, Wolff, Rath, and Fluharty (67). Figure 17 depicts the rapidity with which sequestered red cells may be lysed and their products reutilized. However, comparison of Figures 16 and 17 reveals that in normal subjects the iron released from red cells sequestered in the spleen may be delayed in its reutilization by as much as 16 or 18 hours longer than iron released by intravascular hemolysis. The Fe^{59} studies also indicate that the injurious effect of antibody sensitization on reticulocytes (the only Fe^{59}-labelled cells) is similar to that on mature red cells. Finally, it is of interest to note that the anti-D sensitized red cells (Fe^{59}-labelled reticulocytes) were destroyed very rapidly in a patient with active, severe, acquired hemolytic anemia (Figure 18), whose pathologically sensitized red cells were destroyed with similar speed (at least initially) when transfused into other subjects (Figure 11). Although Figure 11 suggests that on reinjection the patient's pathologically sensitized Cr^{51}-labelled red cells competed with unlabelled red cells in becoming sequestered, it appears that the patient's spleen retained an ample reserve capacity for destroying quickly the more severely sensitized (anti-D coated) red cells.

The observations cited above indicate that following their sequestration by the action of nonhemolytic antibodies, sensitized red cells are catabolized in approximately the following time sequence: 1) The cells are hemolyzed within a few minutes; 2) the released hemoglobin is converted to bilirubin (largely *in situ*) within one to two hours; 3) and the derived iron is available to the bone marrow for reutilization within six to eight hours.

Since lysis takes place within a few minutes of sequestration, it is highly unlikely that it is brought about by the mechanisms of red cell swelling (68) or degeneration of the red cell membrane (69, 70) secondary to stasis and hemoconcentration *per se*, albeit such a sequence probably occurs in hereditary spherocytosis (54–57, 68). Even the theory that sequestered red cells provoke by vascular obstruction and ischemia the release of injurious substances from the tissues (14) seems inadequate to account for the rapidity of action noted here. The search for preformed lysins of physiologic significance in the spleen and other tissues (71–75) has proved a difficult one, not as yet productive of a clear answer. Studies in progress in this laboratory indicate that granulocytopenia and monocytopenia are associated with the lysis of red cells coated with incomplete antibodies and that the leukocytes of subjects hyperimmunized to the D antigen may adhere promptly to type D red cells and later phagocytose some of these red cells (49). That the fixed macrophages of the reticuloendothelial system may react to sensitized red cells similarly or even more strikingly (17), and that lysis, with or without phagocytosis, of trapped sensitized red cells may supervene seems likely. The lining cells of liver sinusoids have been observed to phagocytose protein-coated carbon particles within a fraction of a second (76, 77); therefore, it seems not unreasonable to suppose that such phagocytes may engulf and possibly hemolyze trapped sensitized red cells within a few minutes.

The injection of Cr^{51}-labelled normal red cells into normal subjects with circulating isoagglutinins, but without demonstrable isohemolysins, active against the A or B antigens of the injected cells, led to a more abrupt removal of the injected cells than was observed with sensitized cells; and there rapidly appeared moderate levels of hemoglobin and of Cr^{51} in the plasma (Figure 2). The correspondingly abrupt appearance of high hepatic radioactivity contrasted with the slower accumulation of Cr^{51} by the liver following injection of Cr^{51}-labelled hemoglobin solution. In subjects with demonstrable isohemolysins in addition to isoagglutinins, injection of incompatible red cells produced similar changes, except for the even greater rapidity of red cell destruction, higher and more immediate peak levels of plasma hemoglobin and Cr^{51}, and smaller and more gradual Cr^{51} uptakes by the subjects' livers. It is possible that in those recipients not manifesting hemolysins in their plasma this prompt hemoglobinemia arises from a direct local action of isohemolysins present in certain tissues. The findings cited above indicate also that those red cells not directly hemolyzed were sequestered, probably as agglutinates, and that this occurred largely in the liver. Similarly, as noted above, red cells agglutinated *in vitro* with anti-B serum and reinjected into the donor were observed to lodge in the donor's liver, albeit temporarily. Again, in a study not reported in detail here, the reinjection into a normal subject of Cr^{51}-labelled red cells which had been moderately agglutinated *in vitro* with $CrCl_3$ led to their abrupt, although temporary, sequestration by the subject's liver as indicated by body surface radioactivity. In a dog the reinjection of the animal's red cells more strongly agglutinated *in vitro* with $CrCl_3$ led to sequestration by the lung, and to a lesser extent by the liver.

Together these observations involving moderate or strong agglutination indicate the relative lack of participation of the spleen in the sequestration process, perhaps because of the greater blood flow through other filters efficient against coarse red cell aggregates. The tendency of such cells to lodge in the lung and the liver presumably depends in part upon the size of the agglutinates, the greater blood flow of these organs and, in the case of the lung, its position proximal in the circulation to the site of injection. The filtration of strongly agglutinated red cells by proximate capillary beds seems logically compatible with the manifest inability of such cell aggregates to traverse the capillary beds of various tissues. Nevertheless, the evidence of a tendency of agglutinating antibodies to produce red cell sequestration specifically in the liver rather than in the lung is striking. Hepatic congestion with red cell agglutinates was histologically observed in intact animals by Pearce (22); and later the sequestering process was observed directly (14) during low pressure perfusions of the livers of dogs with dog blood mixed *in vitro* with weakly agglutinating concentrations of anti-dog red cell immune serum. Recently, McKay and co-workers (78) has demonstrated the appearance of multiple minute mesenteric "thrombi" and pseudomembranous enterocolitis following the intra-aortic injection of incompatible red cells into

dogs in shock. This effect is possibly attributable to the phenomenon cited above of agglutinative obstruction in a low pressure perfusing system. Experimental hemolytic anemias in fowl, produced by cationic copper, a potent red cell agglutinating agent, are also associated with hepatic congestion with aggregates of red cells (79). Indeed, patients with acquired hemolytic anemia and frank autoagglutination may develop tender, enlarged livers, which may well be on this basis, for in one such patient, reported elsewhere (33), the liver was found to sequester actively Cr^{51}-labelled autogenous red cells.

The anatomic features of the hepatic circulation which may especially adapt this organ for the sequestration of red cell agglutinates have been described by Knisely, Bloch, Warner, and Piovella (76, 77, 80). By direct microscopic visualization of the living amphibian and mammalian liver, these authors have demonstrated that the liver, like the spleen, has sinusoidal structures permitting the trapping and hemoconcentration of blood. Bloch (77, 80) also observed the rapid appearance of red cell aggregates in the portal venules following intra-portal injection of anti-red cell serum, and shortly thereafter the retention and concentration of such aggregates in the liver sinusoids was evident. It is of interest that very little phagocytosis of these "passively" agglutinated red cells was observed and that, as in human subjects given red cells previously agglutinated with complete antibodies, many of the sequestered, agglutinated red cells appeared to return to the circulation within a few hours. It seems reasonable to ascribe to the liver a function as a filter most effective in trapping particles of somewhat larger size than those easily discriminated by the spleen. There is less clinical information to suggest pathological exaggerations of this filtering action of the liver on blood cells than is the case with the diseased spleen. However, a troublesome aspect of the natural course of acquired hemolytic anemia associated with red cell sensitization is the frequent tendency of hematologic relapses to occur several months after successful splenectomy. In studies of two such patients with acquired hemolytic anemia in relapse the authors have observed hepatomegaly and unusually high hepatic radioactivity after the injection of Cr^{51}-labelled autogenous red cells. Thus, one may conceivably face a clinical phenomenon of "hyper-hepatism," which may only appear or become manifest after removal of the spleen. Similarly, in rare instances, patients with hereditary spherocytosis have been reported to undergo hematologic relapses within a few months after splenectomy and to develop enlarged, tender livers engorged with red cells (81, 82).

As in the studies of ABO isoantibodies above, it may be difficult to exclude the presence *in vivo* either of circulating hemolysins in subliminal concentrations or of high local concentrations of isohemolysins. That immune incompatibility may exist *in vivo* which is not demonstrable *in vitro* has been reported in dogs (17) and in man (83). A further observation to this effect, with additional features, is recorded in Figure 20. A patient with idiopathic acquired hypogammaglobulinemia had a serum gamma globulin concentration of only 20 to 30 mg. per cent. Although her blood was type A, prolonged incubation of large volumes of her fresh serum with type B red cells failed to cause hemolysis, agglutination, or sensitization of these cells. Nevertheless, Cr^{51}-labelled type B red cells were destroyed at an accelerated rate in her circulation, after a delay of two to three days, and this destruction was attended by pronounced splenic sequestration. The sigmoidal red cell survival curve produced resembles that seen in certain of the occult transfusion incompatibilities cited above (17, 83). The initial delay might be interpreted either as a delay required for the stimulation of antibody formation, or for the injected cells to absorb sufficient amounts of a scarce antibody to cause their agglutination or sensitization. To explore these alternative possibilities this hypogammaglobulinemic patient was then given several small weekly injections of type B red cells and thereafter a survival study of Cr^{51}-labelled type B red cells was repeated. At this time these "incompatible" red cells survived almost normally, without abnormal splenic sequestration, a finding consistent with the second interpretation.

There is dispute as to whether the red cells of patients with acquired hemolytic anemia are coated with an "autoantibody" that has developed specifically against the red cells as antigen. Nevertheless, red cells sensitized with a specific antibody, with a drug-antibody complex (84), or even with a nonimmunologic metallo-protein complex (48),

behave similarly *in vitro* and *in vivo*. Examples of acquired hemolytic anemias associated with each of the classic types of "immune" red cell alteration have been observed in which it is clear that specific antibodies had not developed against the unaltered red cell acting as antigen. There have been reports of hemolytic anemias in man caused by: a complement-requiring hemolysin associated with quinidine sensitivity (85); intravenous injection of a metallic cation (Cu^{++}) which strongly agglutinates red cells (86); and red cell sensitization associated with sensitivity to fuadin (84). Thus it appears that the mode of destruction of the red cell does not fundamentally depend upon the specific mechanism of "immune" alteration but chiefly upon whether the cell has been sensitized to the action of complement, whether the cell is agglutinated by the "antibody," or whether it is only agglutinable in the presence of certain co-factors. Based on the studies described above a schema is presented in Figure 21, depicting the several pathways of red cell destruction by antibodies and by the various antibody-like agents encountered in idiopathic acquired hemolytic anemia.

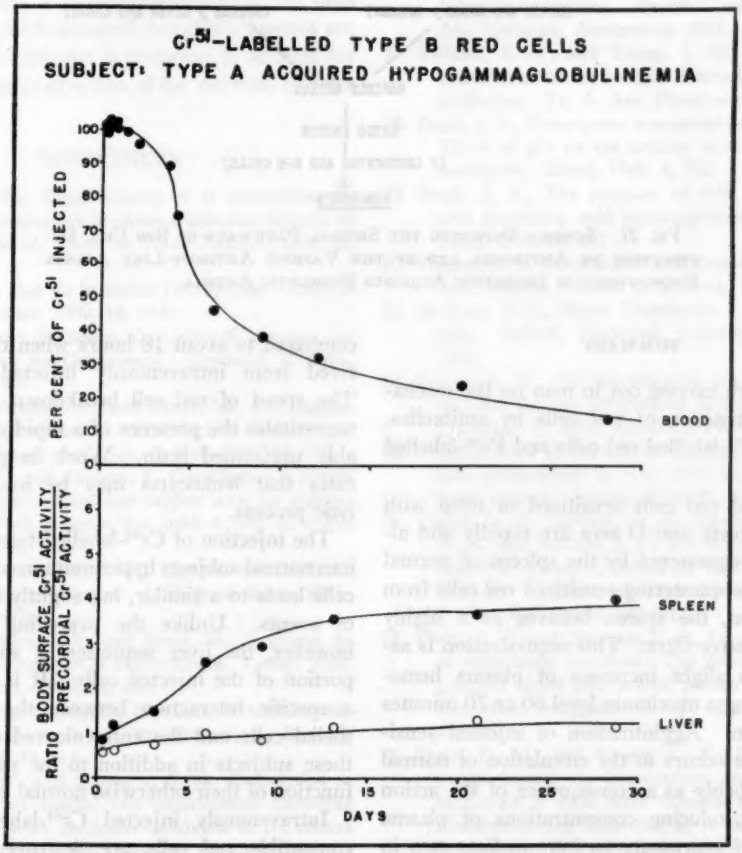

FIG. 20. THE DESTRUCTION OF NORMAL TYPE B RED CELLS IN A TYPE A PATIENT WITH ACQUIRED HYPOGAMMAGLOBULINEMIA AND NO ISOANTIBODIES DEMONSTRABLE *In Vitro*

After a delay of almost two days, a rapid phase of circulating labelled red cell disappearance and of splenic red cell sequestration set in, with gradual slowing of both processes thereafter. Subsequent attempts to demonstrate anti-B antibody *in vitro* were again not fruitful.

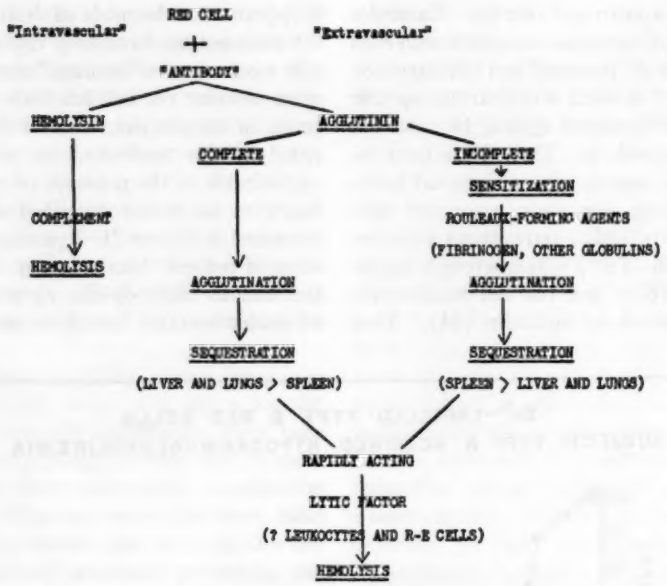

FIG. 21. SCHEMA DEPICTING THE SEVERAL PATHWAYS OF RED CELL DESTRUCTION BY ANTIBODIES AND BY THE VARIOUS ANTIBODY-LIKE AGENTS ENCOUNTERED IN IDIOPATHIC ACQUIRED HEMOLYTIC ANEMIA

SUMMARY

Studies were carried out in man on the mechanisms of destruction of red cells by antibodies, employing Cr^{51}-labelled red cells and Fe^{59}-labelled reticulocytes.

Cr^{51}-labelled red cells sensitized *in vitro* with potent incomplete anti-D sera are rapidly and almost totally sequestered by the spleens of normal subjects. In sequestering sensitized red cells from the circulation, the spleen behaves as a highly proficient, passive filter. This sequestration is associated with slight increases of plasma hemoglobin reaching a maximum level 60 or 70 minutes after injection. Agglutination of injected sensitized red cells occurs in the circulation of normal subjects, probably as a consequence of the action of rouleaux-producing concentrations of plasma globulins, and represents an intermediate step in the mechanism of sequestration. These red cells are hemolyzed within a few minutes of their sequestration and hemoglobin breakdown occurs largely *in situ*. Reappearance in circulating red cells of the iron released from sensitized red cells begins after a delay of about 30 to 36 hours as compared to about 18 hours when the iron is derived from intravenously injected hemoglobin. The speed of red cell breakdown in the spleen necessitates the presence of a rapidly acting, probably preformed lysin. Work in progress indicates that leukocytes may be involved in this lytic process.

The injection of Cr^{51}-labelled type D or C cells into normal subjects hyperimmunized against these cells leads to a similar, but slightly faster, pattern of events. Unlike the preceding observations, however, the liver sequesters a substantial proportion of the injected cells. It is probable that a specific interaction between the reticuloendothelial cells and the antigenic red cells exists in these subjects in addition to the passive filtering function of their otherwise normal spleens.

Intravenously injected Cr^{51}-labelled ABO-incompatible red cells are destroyed abruptly in subjects with isoagglutinins but no demonstrable isohemolysins. This is associated with prominent hepatic sequestration and moderate hemoglobinemia. In subjects with both agglutinins and demonstrable hemolysins red cell destruction is virtually instantaneous and presumably occurs in

the general circulation, with prompt marked hemoglobinemia and slower, less pronounced hepatic accumulations of Cr^{51}. It appears that virtually all normal persons possess some isohemolysins for ABO-incompatible red cells, even when such hemolysins are not demonstrable *in vitro* by standard procedures. Generally, however, isoagglutination with hepatic sequestration is the chief mechanism of destruction of transfused ABO-incompatible red cells. In man frank red cell agglutination produced in diverse ways was uniformly associated with a predominantly hepatic sequestration of the cells.

Studies of the analogous behavior of red cells from patients with acquired hemolytic anemia are presented and a schema is proposed to account for the pathways of destruction of the red cells of these patients.

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ELECTROLYTE AND WATER EXCRETION IN ARTERIAL HYPERTENSION. I. STUDIES IN NON-MEDICALLY TREATED SUBJECTS WITH ESSENTIAL HYPERTENSION [1]

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In a preliminary investigation it was reported that abnormally high rates of sodium excretion followed the infusion of hypertonic saline in untreated patients with essential hypertension (1). Furthermore, it was noted that several oral drugs commonly employed in the treatment of hypertension usually modified this pattern of renal sodium excretion. The present report describes further work on sodium excretion in hypertensive patients and in general agrees with previous reports of other authors (2, 3). In addition, it describes studies made in an attempt to clarify the disturbance of sodium excretion in hypertension and its relationship to renal function, renal hemodynamics, salt intake, and splanchnic sympathetic activity.

MATERIAL AND METHODS

The eight normotensive subjects in Group I had no history of hypertension and were considered normal with the exception of D. R. who had bronchial asthma in remission at the time of the study, and M. G. who had asymptomatic chronic pyelonephritis.

The nine subjects in Group II were known to have essential hypertension for at least five years. They were all compensated and had no history of congestive heart failure. However, five subjects in Group II had hypertensive heart disease, with an enlarged left ventricle as indicated by the electrocardiogram and chest roentgenogram.

The eight subjects in Group III, who also had essential hypertension without cardiac decompensation, had been surgically treated more than two years before the study with a bilateral thoracolumbar sympathectomy. Patient T. S., in addition, had had a total bilateral adrenalectomy for malignant hypertension. Four patients of the group had definite hypertensive heart disease.

Dietary intake of salt was not restricted. All hypertensive patients had been without antihypertensive drug treatment for at least three months prior to the study. The average surface areas of patients in the three groups were comparable.

All subjects were studied without sedative medication in the morning, in the post-absorptive state while lying supine. Throughout the study the subjects drank 100 ml. of water every half-hour. Urine was collected through an indwelling bladder (Foley) catheter. At the end of each collection the bladder was washed out with 20 ml. of distilled water and was then completely evacuated with air. After standard priming doses, a sustaining infusion containing inulin (3.3 per cent) and para-aminohippurate (1 per cent) in 3.5 per cent glucose in water was started and given at the rate of 1.2 ml. per minute by means of calibrated drip. Every 20 minutes arterial blood samples were obtained for the determination of inulin, para-aminohippuric acid, sodium, potassium and hematocrit. Following the control period which consisted of three to four urine collections, each one being for 10 to 15 minutes, 300 ml. of 5 per cent sodium chloride was administered intravenously at the rate of 10 to 12 ml. per minute. At the end of the infusion a urine collection designated as "5 per cent NaCl" was made. Thereafter, at least three urine collections were obtained in the "recovery" period at intervals of 15 minutes. In a few subjects following the "recovery" period, Pitressin® was injected intravenously in a single dose of 40 to 100 milliunits. After the injection three more urine collections were made in the "Pitressin®" period at intervals of 15 minutes.

Blood and urine concentrations of para-aminohippuric acid were measured by the method of Goldring and Chasis (4), and inulin was determined by the method of Roe, Epstein, and Goldstein (5). Serum and urinary sodium and potassium were determined by the internal standard flame photometer (lithium standard). The hematocrit was determined in Wintrobe tubes. Measurements of brachial arterial pressure were obtained with an electromanometer (Sanborn) and recorded by direct-writing oscillograph.

RESULTS

The renal responses to intravenous 5 per cent sodium chloride in the normotensive and hypertensive groups are summarized in Table I. The

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statistical analysis of the responses are given in Table II.

PAH clearance (renal plasma flow)

The control renal plasma flows in the hypertensive groups were not significantly different from those in the normotensive group. All three groups had significant increases in renal plasma flow during the infusion of 5 per cent sodium chloride. In only the non-splanchnicectomized hypertensive group were the increases significantly prolonged into the recovery period. Otherwise the changes in renal plasma flow during and following the saline infusion were not significantly different in the three groups.

Inulin clearance (glomerular filtration rate)

The glomerular filtration rates at rest did not differ significantly between the hypertensive and normotensive groups. During and following the saline infusion, the splanchnicectomized hypertensive group like the normotensive group had no significant increases in glomerular filtration rate although the *untreated* hypertensive group had small but significant increases in this function. Otherwise, the changes in glomerular filtration rate caused by the hypertonic saline were not significantly different in the groups.

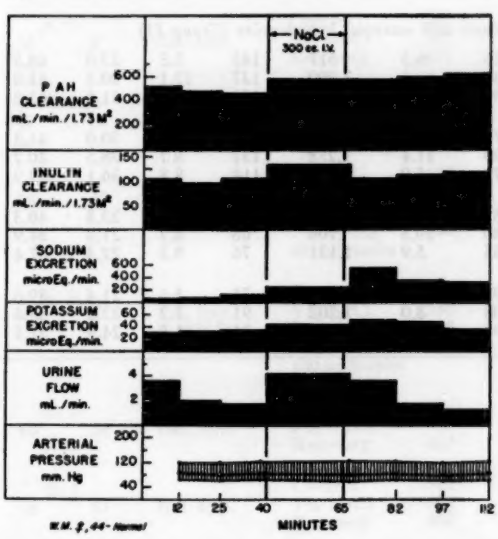

FIG. 1. RENAL RESPONSES TO INTRAVENOUS HYPERTONIC SALINE IN A NORMOTENSIVE INDIVIDUAL

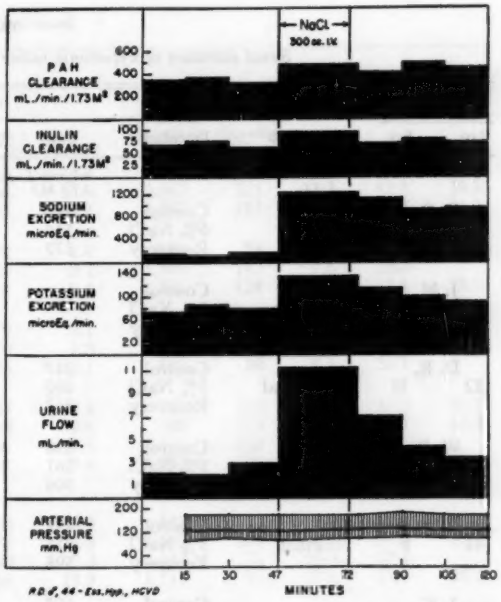

FIG. 2. RENAL RESPONSES TO INTRAVENOUS HYPERTONIC SALINE IN A SUBJECT WITH ESSENTIAL HYPERTENSION

Sodium excretion

The control sodium excretion in the untreated hypertensive group but not in the splanchnicectomized hypertensive group was significantly higher than in the normotensive group. Although all three groups showed significant increases in sodium excretion during and following the infusion of 5 per cent sodium chloride, the increases were significantly greater in the hypertensive groups than in the normotensive group (Figures 1, 2 and 3). Included in the splanchnicectomized hypertensive group was an adrenalectomized subject who also had an abnormally high rate of sodium excretion after the saline infusion (Figure 4). The mean increase in sodium excretion in the splanchnicectomized hypertensive group was not as great as in the non-splanchnicectomized hypertensive group.

Ratio of excreted sodium to filtered sodium (E/F sodium)

The calculated ratio of excreted sodium to filtered sodium (E/F sodium) both before and after

TABLE I

Renal excretion of hypertonic saline in normotensive individuals (Group I)

Patient Age	Sex	Diagnosis‡	Procedure	C_{PAH}	C_{IN}	UV	$U_{Na}V$	$U_{K}V$	$\frac{E}{F}$ Na*	$\frac{E}{F}$ K†	Hematocrit (arterial)
				ml./min./ 1.73 M.²	*ml./min./ 1.73 M.²*	*ml./min.*	*MicroEq./ min.*	*MicroEq./ min.*	%	%	%
V. B. 31	F	Normal	Control	447	90	2.9	155	90	1.2	25.6	42.0
			5% NaCl	519	97	2.7	260	88	1.8	22.0	38.0
			Recovery	572	90	1.5	366	81	2.7	23.7	38.4
J. M. 27	F	Normal	Control		106	3.4	84	233	0.5		42.5
			5% NaCl		100	2.0	184	158	1.2		37.8
			Recovery		106	1.5	243	223	1.5		38.2
D. R. 52	M	Bronchial asthma	Control	347	96	6.9	188	58	1.4	13.4	54.9
			5% NaCl	407	123	7.6	391	78	2.2	14.1	50.1
			Recovery	367	126	1.7	328	41	1.8	7.2	51.9
W. P. 32	F	Normal	Control	498	98	2.0	213	65	1.6	15.9	42.2
			5% NaCl	561	109	2.8	526	54	3.4	13.3	
			Recovery	504	100	4.1	599	40	4.4	10.0	38.4
W. M. 44	F	Normal	Control	481	102	2.5	98	33	0.7	8.1	36.3
			5% NaCl	575	130	4.2	244	43	1.3	8.5	32.3
			Recovery	598	112	2.3	425	46		10.5	33.9
J. K. 47	M	Normal	Control	505	98	6.3	60	84	0.5	24.6	46.0
			5% NaCl	579	99	7.9	218	94	1.5	26.4	41.5
			Recovery	451	94	1.1	376	61	1.1	21.3	41.7
M. G. 62	F	Chronic pyelonephritis	Control	182	54	2.6	81	43	1.6	18.1	48.4
			5% NaCl	211	57	3.3	319	60	3.9	23.9	43.0
			Recovery	234	56	1.8	306	59	3.8	24.0	44.4
C. J. 38	F	Normal	Control	632	101	11.3	101	90	0.7	22.6	42.7
			5% NaCl	598	91	12.5	168	52			
			Recovery	623	102	1.3	275	41	1.1	10.0	40.6
Renal excretion of hypertonic saline in individuals with essential hypertension (Group II)											
K. B. 35	M	Ess. hyp.	Control	565	126	6.3	617	145	3.5	23.0	46.5
			5% NaCl	744	130	12.8	2,290	137	12.1	20.3	42.6
			Recovery	789	135	7.7	1,561	152	7.9	21.6	43.2
R. D. 44	M	Ess. hyp. H.C.V.D.	Control	351	73	2.6	152	87	1.4	30.0	44.3
			5% NaCl	484	99	11.4	1,258	137	8.7	38.5	40.2
			Recovery	462	79	5.0	1,014	114	8.8	36.1	39.9
D. C. 36	F	Ess. hyp. H.C.V.D.	Control	309	81	12.4	601	77	5.5	23.8	40.3
			5% NaCl	303	80	10.5	706	68	6.2	21.3	34.9
			Recovery	355	85	5.9	1,131	76	9.5	22.3	37.4
D. F. 45	F	Ess. hyp.	Control	390	91	4.7	723	74	5.6	21.4	39.6
			5% NaCl	530	108	8.0	1,202	91	7.2	22.2	35.5
			Recovery	468	96	7.7	1,661	91	11.5	24.9	37.5

* $\frac{E}{F}$ Na = $\frac{\text{sodium excreted}}{\text{sodium filtered}}$.

† $\frac{E}{F}$ K = $\frac{\text{potassium excreted}}{\text{potassium filtered}}$.

‡ Ess. hyp. = Essential hypertension.

H.C.V.D. = Hypertensive cardiovascular disease.

§ Previously adrenalectomized.

TABLE I—*Continued*

Patient Age	Sex	Diagnosis‡	Procedure	C_{PAH}	C_{IN}	UV	$U_{Na}V$	$U_{K}V$	$\frac{E}{F}$ Na*	$\frac{E}{F}$ K†	Hematocrit (arterial)
				ml./min./ 1.73 M²	*ml./min./ 1.73 M²*	*ml./min.*	*MicroEq./ min.*	*MicroEq./ min.*	%	%	%
I. P.			Control	467	131	4.8	765	104	4.5	22.6	43.3
42	M	Ess. hyp.	5% NaCl	659	142	13.0	2,387	211	11.3	42.4	39.8
		H.C.V.D.	Recovery	624	133	12.2	2,364	163	12.1	35.8	41.0
C. C.			Control	458	106	8.0	250	78	1.6	17.4	38.3
48	F	Ess. hyp.	5% NaCl	643	128	8.2	1,340	143	7.0	28.5	
		H.C.V.D.	Recovery	625	132	9.3	1,726	128	8.7	22.4	38.0
R. O.			Control	322	79	9.1	141	121	1.2	40.3	40.1
50	M	Ess. hyp.	5% NaCl	338	79	8.0	975	139	8.0	51.7	37.8
		H.C.V.D.	Recovery	320	85	4.1	802	80	6.5	26.1	
W. M.			Control	443	127	8.4	216	153	1.2	31.6	46.1
40	M	Ess. hyp.	5% NaCl	518	148	9.6	686	229	3.1	38.7	44.3
			Recovery	464	131	8.6	1,276	214	6.8		
			Pitressin® 40 milliunits	494	131	7.4	1,032	299	5.4	50.7	44.7
J. W.			Control		60	3.9	522	72	6.2	30.0	39.5
50	M	Ess. hyp.	5% NaCl		78	14.5	1,682	106	14.5	35.9	37.4
		H.C.V.D.	Recovery		60	7.3	1,467	74	16.5	30.9	
			Pitressin® 80 milliunits		68	10.9	1,711	98	17.2	28.7	38.4
Renal excretion of hypertonic saline in splanchnicectomized individuals with essential hypertension (Group III)											
V. V.			Control	537	118	1.9	176	57	1.5	12.1	39.4
37	F	Ess. hyp.	5% NaCl	756	138	6.7	1,013	85	4.9	15.4	36.8
		H.C.V.D.	Recovery	760	129	5.1	1,092	70	5.7	13.6	37.7
I. R.			Control	402	103	1.5	137	119	0.9	23.1	44.3
49	M	Ess. hyp.	5% NaCl	457	120	5.6	754	182	4.2	30.3	41.6
		H.C.V.D.	Recovery	451	118	5.3	867	147	5.1	25.0	41.7
A. G.			Control	527	100	7.8	599	50	4.4	12.5	36.5
36	F	Ess. hyp.	5% NaCl	567	106	17.0	1,530	60	10.0	13.8	34.6
			Recovery	471	89	6.7	1,270	52	10.0	13.9	
T. S.§			Control	108	28	2.5	292	37	7.3	25.5	49.0
49	M	Ess. hyp.	5% NaCl	160	40	7.2	948	74	16.5	43.0	46.2
		H.C.V.D.	Recovery	110	33	6.1	867	64	18.7	42.1	46.8
A. L.			Control	425	119	12.7	1,164	82	6.9	18.7	44.9
60	M	Ess. hyp.	5% NaCl	381	103	14.3	1,758	87	11.4	26.4	41.8
		H.C.V.D.	Recovery	438	115	8.8	1,614	79	10.0	20.3	
			Pitressin® 40 milliunits	450	119	7.4	1,402	98	2.6		43.4
N. K.			Control	374	72	2.4	262	81	2.5	28.8	52.7
49	F	Ess. hyp.	5% NaCl	550	83	6.0	746	127	5.9	40.3	49.0
		H.C.V.D.	Recovery	508	87	7.8	1,051	125	8.1	36.8	
			Pitressin® 100 milliunits	537	97	8.0	1,182	130	8.1	31.3	49.1
R. T.			Control	332	84	1.5	209	87	1.8	20.7	42.1
40	M	Ess. hyp.	5% NaCl	441	97	4.8	871	114	6.2	23.5	38.2
			Recovery	455	97	6.2	1,205	107	8.5	22.6	41.0
N. I.			Control	322	83	5.2	39	32	0.3	8.8	47.5
58	M	Ess. hyp.	5% NaCl	395	78	5.1	848	74	7.5	21.1	44.0
			Recovery	407	96	5.9	913	77	6.6	17.9	44.8

TABLE II

Mean data and statistical analysis

	Control	5% NaCl	5% NaCl − Control	p	Recovery	Recovery − Control	p
			PAH clearance *ml./min./1.73M.²*				
Normotensive group	442 ± 54	493 ± 53	+51 ± 16	0.02	478 ± 53	+37 ± 25	0.2
Hypertensive group	413 ± 31	527 ± 54	+114 ± 27	<0.01	513 ± 55	+100 ± 28	<0.01
Mean difference*	−29 ± 62	+34 ± 76	+63 ± 32		+35 ± 76	+63 ± 37	
p	0.6	0.7	0.07		0.6	0.1	
Splanchnicectomized hypertensive group	378 ± 48	463 ± 61	+85 ± 29	0.02	450 ± 62	+72 ± 31	0.06
Mean difference*	−63 ± 72	−29 ± 81	+34 ± 33		−28 ± 82	+35 ± 40	
p	0.4	0.7	0.3		0.7	0.4	
			Inulin clearance *ml./min./1.73M.²*				
Normotensive group	93 ± 6	101 ± 8	+8 ± 5	0.2	98 ± 7	+5 ± 4	0.2
Hypertensive group	97 ± 9	110 ± 9	+13 ± 3	<0.01	104 ± 10	+7 ± 2	0.03
Mean difference*	+4 ± 10	+9 ± 12	+5 ± 6		+6 ± 12	+2 ± 4	
p	0.7	0.4	0.3		0.6	0.7	
Splanchnicectomized hypertensive group	88 ± 10	95 ± 10	+7 ± 4	0.1	95 ± 10	+7 ± 3	0.08
Mean difference*	−5 ± 12	−5 ± 13	0 ± 6		−3 ± 13	+2 ± 5	
p	0.7	0.7	>0.9		0.8	0.7	
			$U_{Na}V$ *MicroEq./min.*				
Normotensive group	123 ± 20	289 ± 42	+166 ± 29	<0.01	365 ± 39	+242 ± 32	<0.01
Hypertensive group	443 ± 84	1391 ± 207	+948 ± 176	<0.01	1444 ± 154	+1001 ± 115	<0.01
Mean difference*	+320 ± 86	+1103 ± 211	+782 ± 179		+1080 ± 159	+759 ± 119	
p	<0.01	<0.01	<0.01		<0.01	<0.01	
Splanchnicectomized hypertensive group	360 ± 129	1058 ± 133	+699 ± 52	0.01	1110 ± 89	+750 ± 64	<0.01
Mean difference*	+237 ± 130	+770 ± 140	+532 ± 60		+745 ± 98	+508 ± 72	
p	0.09	0.01	0.01		0.01	0.01	
			$U_{K}V$ *MicroEq./min.*				
Normotensive group	87 ± 22	78 ± 13	−9 ± 11	0.5	74 ± 22	−13 ± 7	0.1
Hypertensive group	101 ± 10	140 ± 17	+39 ± 13	0.02	121 ± 16	+20 ± 11	0.1
Mean difference*	+14 ± 24	+62 ± 22	+48 ± 17		+47 ± 27	+33 ± 13	
p	0.6	0.01	0.02		0.1	0.03	
Splanchnicectomized hypertensive group	68 ± 10	100 ± 14	+32 ± 7	<0.01	90 ± 12	+22 ± 6	<0.01
Mean difference*	−19 ± 24	+22 ± 19	+41 ± 13		+16 ± 25	+35 ± 10	
p	0.4	0.3	<0.01		0.5	0.01	
			UV *ml./min.*				
Normotensive group	4.7 ± 1.1	5.4 ± 1.3	+0.6 ± 0.4	0.1	1.9 ± 0.3	−2.8 ± 1.3	0.08
Hypertensive group	6.7 ± 1.0	10.7 ± 0.8	+4.0 ± 1.6	0.03	7.5 ± 0.8	+0.8 ± 1.4	0.6
Mean difference*	+2.0 ± 1.5	+5.3 ± 1.5	+3.4 ± 1.6		+5.6 ± 0.9	+3.6 ± 2.0	
p	0.2	<0.01	0.05		<0.01	0.08	
Splanchnicectomized hypertensive group	4.4 ± 1.4	8.3 ± 1.6	+3.9 ± 1.0	<0.01	6.5 ± 0.4	+2.0 ± 1.1	0.1
Mean difference*	−0.3 ± 1.8	+2.9 ± 2.1	+3.3 ± 1.0		+4.6 ± 0.6	+4.9 ± 1.8	
p	0.9	0.2	<0.01		<0.01	0.02	
			Mean arterial pressure *mm. Hg*				
Normotensive group	87 ± 2	89 ± 3	+2 ± 3	0.6	89 ± 2	+2 ± 2	0.4
Hypertensive group	144 ± 5	144 ± 5	0 ± 2	>0.9	144 ± 5	0 ± 1	>0.9
Mean difference*	+57 ± 6	+55 ± 6	−2 ± 4		+55 ± 5	−2 ± 2	
p	<0.01	<0.01	0.6		<0.01	0.5	
Splanchnicectomized hypertensive group	152 ± 11	156 ± 12	+3 ± 3	0.3	151 ± 11	−1 ± 2	0.6
Mean difference*	+66 ± 11	+67 ± 12	+1 ± 4		+62 ± 11	−3 ± 3	
p	<0.01	<0.01	0.8		<0.01	0.3	

* Refers to the difference between the normotensive and the designated hypertensive group.

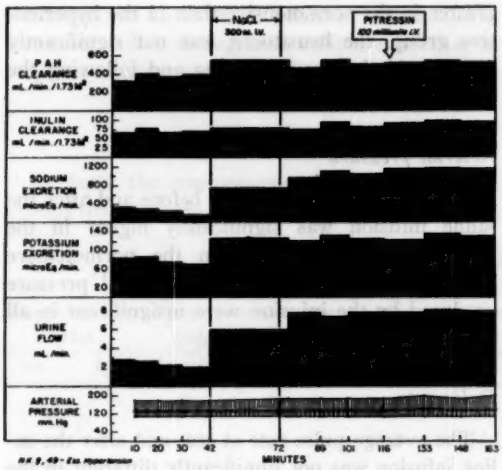

FIG. 3. RENAL RESPONSES TO INTRAVENOUS HYPERTONIC SALINE IN SPLANCHNICECTOMIZED HYPERTENSIVE SUBJECT

the infusion of saline was significantly higher in the hypertensive groups than in the normotensive group. The increases in this ratio after the infusion, which were significant in all groups, were likewise significantly greater in the hypertensive groups than in the normotensive group.

Correlations of sodium excretion

In analyzing the groups all together (Table III), sodium excretion before and after the saline infusion was significantly correlated (statistically) with the control arterial pressure. Increases in sodium excretion following intravenous 5 per cent sodium chloride were likewise significantly correlated with changes in renal plasma flow but not with changes in glomerular filtration rate, however. They were not significantly correlated with the control renal plasma flow or with the control glomerular filtration rate.

In analyzing the small groups separately, the control sodium excretion did not show a significant statistical correlation with the control arterial pressure. Increases in sodium excretion produced by the saline infusion also were not correlated with the control arterial pressure, renal plasma flow and glomerular filtration rate, or with the changes in these functions which occurred after the infusion of saline.

Potassium excretion

The control excretion of potassium in the hypertensive groups was not significantly different from that in the normotensive group. During and following the infusion of 5 per cent saline, the average potassium excretion in the normotensive group decreased slightly but insignificantly whereas in both hypertensive groups it increased slightly. The latter increases were statistically significant except for those in the untreated hypertensive group during the saline infusion.

Ratio of excreted potassium to filtered potassium (E/F potassium)

The calculated ratio of excreted potassium to filtered potassium (E/F potassium) during the control period was significantly higher in the untreated but not in the splanchnicectomized hypertensive group than in the normotensive group. This ratio did not change in the normotensive

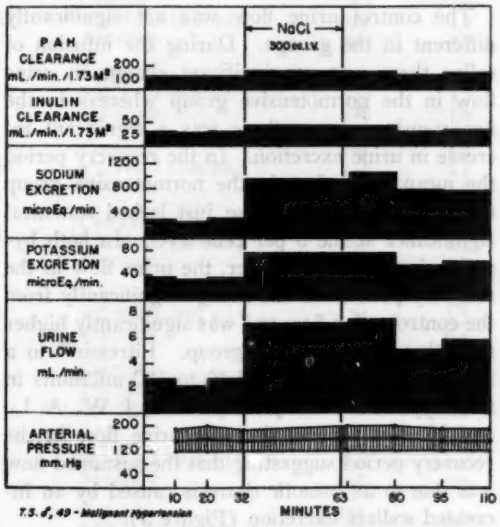

FIG. 4. RENAL RESPONSES TO INTRAVENOUS HYPERTONIC SALINE IN A SPLANCHNICECTOMIZED AND ADRENALECTOMIZED HYPERTENSIVE SUBJECT

The subject, who was maintained on 25 mg. of oral cortisone and 5 mg. of a DOCA linguet daily, had an abnormaly high rate of sodium excretion following the infusion of 5 per cent NaCl even though the renal plasma flow and glomerular filtration rate were markedly reduced.

TABLE III

Correlations of sodium excretion

ΔNa excretion* correlated with:	r†	p‡
Control Na excretion	0.301	0.2
Control mean B.P.	0.532	<0.01
Control PAH clearance	0.165	0.5
Control inulin clearance	0.237	0.3
ΔPAH clearance*	0.541	<0.01
ΔInulin clearance*	0.226	0.3
Control Na excretion correlated with control mean B.P.	0.436	0.03

* Changes in function following saline infusion.
† r = Coefficient of correlation.
‡ p = Probability.

group during the infusion but it increased significantly in the hypertensive groups and was significantly higher in these groups than in the control group. In the recovery period the E/F potassium continued to be significantly higher in the non-splanchnicectomized hypertensive group than in the normotensive group.

Urine flow

The control urine flow was not significantly different in the groups. During the infusion of saline there was no significant change in urine flow in the normotensive group whereas in the hypertensive groups there was a significant increase in urine excretion. In the recovery period the mean urine flow in the normotensive group decreased but this decrease just lacked statistical significance at the 5 per cent level. In both hypertensive groups, however, the urine flow in the recovery period did not change significantly from the control urine flow and was significantly higher than that in the control group. Pitressin® in a single intravenous dose of 40 to 100 milliunits in four hypertensive subjects (W. M., J. W., A. L., and N. K.) did not decrease urine flow in the recovery period suggesting that the sustained flow was due to an osmotic diuresis caused by an increased sodium excretion (Figure 3).

Arterial hematocrit

The arterial hematocrit at rest was not significantly different in the groups. After the infusion of hypertonic saline, it significantly decreased in all three groups. Although the decrease in hematocrit during the infusion was significantly greater in the normotensive than in the hypertensive group, the hematocrit was not significantly different in the groups during and following the infusion.

Arterial pressure

The mean arterial pressure before and after the saline infusion was significantly higher in the hypertensive groups than in the normotensive group. The changes in mean arterial pressure produced by the infusion were insignificant in all the groups.

Pulse rate

The average pulse rate at rest and after the saline infusion was not significantly different in the groups and did not change significantly.

Sodium intake and sodium excretion

In Table IV the effect of altering the sodium content of the diet on the excretion of sodium in response to intravenous 5 per cent sodium chloride is summarized.

During sodium restriction a slight and consistent reduction in weight but not in blood pressure occurred. Associated with the changes in diet and weight, the increases in sodium excretion following the infusion of 300 ml. of 5 per cent saline were reduced and were not as great as those found during an unrestricted salt diet. However, sodium excretion was not necessarily reduced to normal by salt restriction in the hypertensive subjects since it remained unequivocally high in at least one (A. L.) of the three subjects. Normotensive subject E. H., who was placed on an 18-gram salt diet, developed slight peripheral edema and a weight gain of six pounds. While on this diet, sodium excretion in response to intravenous hypertonic saline was greater than that observed during a 10-gram salt diet and approached the values found in hypertensive individuals. To determine whether acute increases in body fluid and sodium might augment the excretory response to hypertonic saline in normotensive subjects, isotonic saline was infused at the rate of 22 ml. per minute in patients H. D. and P. G. prior to the administration of 5 per cent sodium chloride. The prior expansion of the extracellular fluid volume

with 1,000 to 1,700 ml. of saline in both subjects, however, failed to augment sodium excretion in response to intravenous 5 per cent sodium chloride.

DISCUSSION

In both the normotensive and hypertensive groups the increases in sodium excretion produced by the infusion of hypertonic saline were associated with increases in the calculated load of filtered sodium. Although the latter changes were not measureably different between the groups, the increases in sodium excretion were greater in both the splanchnicectomized and the non-splanchnicectomized hypertensive groups than in the normotensive group. The differences in sodium

TABLE IV

The effect of salt intake on the excretion of sodium

Patient Age	Sex	Daily NaCl intake	Duration	Weight	Blood pressure	Procedure	$U_{Na}V$	UV
		Gms.	*days*	*pounds*	*mm. Hg*		*MicroEq./min.*	*ml./min.*
W. P. 65	M	15	14	162.0	180/110	Control	871	3.7
						5% NaCl (300 ml.)	1,127	6.8
						Recovery	2,048	10.7
		2	10	160.0	170/120	Control	81	2.0
						5% NaCl (300 ml.)	221	9.3
						Recovery	303	2.5
A. L. 45	M	15	14	158.2	250/150	Control	1,064	6.5
						5% NaCl (300 ml.)	1,640	11.2
						Recovery	1,740	11.5
		2	10	156.0	240/145	Control	40	1.8
						5% NaCl (300 ml.)	525	4.2
						Recovery	560	3.5
I. C. 58	F	10	10	140.0	190/110	Control	242	7.2
						5% NaCl (300 ml.)	836	8.7
						Recovery	878	4.9
		2	10	139.0	190/110	Control	101	5.9
						5% NaCl (300 ml.)	424	9.1
						Recovery	343	2.7
E. H. 42	F	10	9	165.5	130/80	Control	206	6.7
						5% NaCl (300 ml.)	331	4.7
						Recovery	326	1.5
		1	7	162.5	130/76	Control	9	4.8
						5% NaCl (300 ml.)	24	2.7
						Recovery	15	0.6
		18	6	168.5	126/74	Control	235	10.2
						5% NaCl (300 ml.)	569	9.4
						Recovery	939	3.1
H. D. 29	F	10	5	114.5	110/70	Control	117	10.1
						5% NaCl (300 ml.)	267	1.4
						Recovery	442	2.0
		10	5	114.2	110/60	Control	214	3.2
						0.9% NaCl (1,000 ml.)	152	4.5
						5% NaCl (300 ml.)	348	2.9
						Recovery	481	1.9
P. G. 22	F	10	7	161.5	120/70	Control	393	2.9
						5% NaCl (300 ml.)	586	5.8
						Recovery	589	1.9
		10	5	162.0	118/74	Control	106	4.7
						0.9% NaCl (1,700 ml.)	166	4.1
						5% NaCl (300 ml.)	340	2.7
						Recovery	323	1.1

excretion therefore appeared to be due to a difference in the renal tubular handling of sodium. Abnormally high rates of sodium excretion in hypertensive individuals have also been reported to occur during mannitol diuresis (6), suggesting that the hypertensive renal response is not a specific one to the elevated serum sodium produced by intravenous 5 per cent sodium chloride but is a non-specific response to an increase in the effective osmotic pressure of the serum.

The lack of a high degree of correlation between the level of the blood pressure and the renal excretory response to a 5 per cent sodium chloride suggests that in addition to the arterial pressure other factors operate to enhance sodium excretion in patients with essential hypertension. Green and Ellis have been able to relate the renal response of hypertensive individuals to an elevated control sodium excretion (7), but in the present study this relationship was not found to be consistent. Control sodium excretion was considerably higher in untreated hypertensive than in normotensive individuals, but in splanchnicectomized hypertensive subjects the control sodium excretion was not significantly different from that of normotensive subjects. Although these findings indicate that an elevated control sodium excretion is not necessary for large increases in sodium excretion to occur after intravenous 5 per cent sodium chloride, the greater increases of sodium excretion in the untreated hypertensive group than in the sympathectomized hypertensive group suggest that it is capable of augmenting the excretory response to hypertonic saline.

By altering the sodium content of the diet in a small number of hypertensive and normotensive individuals, similar directional changes in sodium excretion following the infusion of 5 per cent sodium chloride appeared to result. These observations might be interpreted to indicate that the excretion of sodium is a function of total body sodium. However, the failure to augment sodium excretion by the prior expansion of the extracellular fluid volume with isotonic saline does not support this hypothesis. Luetscher and Curtis' studies (8) suggest that the observed changes in sodium excretion might be the result of an alteration in adrenal cortical activity. Green and Ellis have presented evidence that significant differences in dietary salt intake may actually exist between normotensive and hypertensive individuals and may account for their different excretory responses to intravenous hypertonic saline (7). The findings in the present study, however, indicate that sodium excretion may be abnormal in hypertensive subjects even though their dietary salt intake is normal.

Primary as well as secondary changes in adrenal cortical activity may affect the renal responses to intravenous 5 per cent sodium chloride. Burnett has found that non-hypertensive patients with adrenal cortical insufficiency (Addison's disease) have an impaired excretory response to intravenous hypertonic saline which is partially corrected by steroid treatment (9). On the other hand, Birchall, Tuthill, Jacobs, Trautman, and Findley have observed that in adrenal cortical hyperfunction (Cushing's syndrome) sodium excretion is abnormally high following an infusion of hypertonic saline. By pre-treating a normotensive individual with cortisone, the same investigators were able to augment sodium excretion following intravenous 5 per cent sodium chloride (3). Although the level of adrenal cortical activity appears to be capable of affecting sodium excretion, the observations in an adrenalectomized hypertensive patient, T. S., who had an increased capacity to excrete sodium while on replacement steroid therapy, suggests that the hypertensive response to intravenous 5 per cent sodium chloride does not necessarily depend upon an increase in adrenal function.

SUMMARY

Both splanchnicectomized and non-splanchnicectomized hypertensive individuals have an increased capacity to excrete sodium as indicated by their excretion of sodium following the infusion of 300 ml. of 5 per cent sodium chloride. The capacity to excrete sodium was significantly correlated with the systemic arterial pressure but not with the control sodium excretion, renal plasma flow and glomerular filtration rate. However, the lack of a high degree of correlation between blood pressure and sodium excretion suggests that additional factors operate to enhance sodium excretion in individuals with essential hypertension. Some of the factors considered were dietary so-

dium intake, extracellular fluid volume, total body sodium and adrenal cortical function.

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RADIOACTIVITY OVER THE SPLEEN AND LIVER FOLLOWING THE TRANSFUSION OF CHROMIUM^{51}-LABELLED ERYTHROCYTES IN HEMOLYTIC ANEMIA [1]

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The beneficial effect of splenectomy in hereditary spherocytosis is well recognized. Selective retention of spherocytes in the spleens of patients with congenital hemolytic anemia was reported by Emerson, Shen, and Castle in 1946 (1). Subsequently, spherocyte "trapping" was demonstrated in spleens removed from patients without this disorder (2, 3). In addition, these abnormal red cells in the spleen have greater osmotic fragility than those in the peripheral blood (1). Ham and Castle first postulated that the spleen increased the osmotic fragility of red cells by intravascular stasis in a manner similar to that produced by sterile incubation of blood (4–7). Recently it has been suggested that the spleen "conditions" spherocytes for early osmotic hemolysis or increases their susceptibility to destruction by the mechanical forces of the circulation (8, 9). Although exact mechanisms of spherocyte destruction remain obscure, splenectomy in congenital hemolytic anemia alleviates the hemolytic process.

The role of the spleen in red cell destruction in acquired hemolytic anemia is less thoroughly understood. Splenic hemosiderosis, erythrophagocytosis, and congestion with red cells suggest the importance of red cell sequestration by this organ in the pathogenesis of many of the acquired hemolytic anemias. The first good response to splenectomy was reported by Micheli in 1911 (10). This operation has since been performed with unpredictable results in diseases having little in common other than hemolytic anemia. Criteria for predicting a favorable result from splenectomy in acquired, or autoimmune, hemolytic anemia have not been provided by conventional laboratory aids.

Determination of relative organ distribution of radioactive erythrocytes is feasible by external scintillation counting (11). Jandl, Greenberg, Yonemoto, and Castle have reported a method for determining organ sites of red cell sequestration using Cr^{51}-labelled erythrocytes (12, 13). They reported splenectomy was beneficial in one patient who had splenic accumulation of labelled red cells.

A method of determining organ localization of Cr^{51}-labelled red cells utilizing the ratio spleen: liver radioactivity as estimated by external scintillation counting was reported from this laboratory in 1955 (14). It was assumed that a high splenic uptake of radioactivity concomitant with an increased rate of Cr^{51} red cell disappearance from the peripheral blood was evidence for abnormal red cell accumulation in the spleen. The present report of red cell survivals and organ counting data was derived from 27 labelled red cell infusions. Splenic uptake of radioactivity in hemolytic anemia was correlated with response to splenectomy.

MATERIALS AND METHODS

Conventional laboratory data

In patients suspected of hemolytic anemia, determinations of hemoglobin, reticulocytes, hematocrit, red blood cell count, red cell fragilities (15), and serum bilirubin (16, 17) were performed by established techniques. The method of Dacie was used for the determination of autohemolysis of red cells (18). Erythroid: myeloid ratios were computed after counting 1,000 nucleated cells in the bone marrow smear. Average fecal urobilinogen in a four-day stool collection was determined by the method of Watson (19), using a Coleman junior colorimeter. One stool urobilinogen determination was semiquantitative (20). When these data showed evidence for hemolytic

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TABLE I

Initial evidence for hemolytic anemia in patients studied by Cr^{51} red cell survival and organ counting

Subject	Family history	Spleen size (*cm.* below costal margin)	Reticulocytes (%)	Hemoglobin (*Gm./100 ml.*)	Hematocrit (%)	No. red cells ($\times 10^6$/*mm.*^{3})	Erythroid: Myeloid ratio	Mean osmotic fragility of red cells (% saline at 50% hemolysis)* Direct	Incubated	48-hr. autohemolysis† (%)	Mechanical fragility‡ (%)	Indirect serum bilirubin (*mg./100 ml.*)	Fecal urobilinogen (*mg./24 hr.*)	Final diagnosis
N. H.	Present	0	7.1	13.0	36.5			0.44	0.58	8.0	9.4	1.1	154 204	Congenital hemolytic anemia
V. H.	Present	0	6.0	13.2	37.0	3.92		0.46	0.61		12.0	1.7	482	Congenital hemolytic anemia
E. Z.	Present	18	9.1	13.4	37.0	4.56		0.46	0.59	21.0	13.0	2.0	1,629	Congenital hemolytic anemia
L. R.	Absent	9	12.4	9.2				0.45	0.59	14.0	30.0	2.5	1,800	Congenital hemolytic anemia
A. K.	Absent	2	21.0	6.6	24.0	2.16	9.7	0.46	0.52	31.0	4.6	2.3	712 693	Paroxysmal nocturnal hemoglobinuria
P. P.	Absent	6	5.1	12.6	35.0	3.96	1.7	0.44	0.52	6.3	7.2	2.2	283	Acquired hemolytic anemia (idiopathic)
L. Y.	Absent	10	37.5	4.2	12.0	1.30	2.3	0.49	0.48	4.6	20.0	2.0	§	Acquired hemolytic anemia; chronic lymphocytic leukemia
L. H.	Absent	8	8.8	10.4	33.0	3.68	1.0			2.6	3.7	1.6	177	Acquired hemolytic anemia (idiopathic)
G. S.	Absent	27	4.7	11.7	38.5	4.20		0.52	0.32	2.7	5.1	1.4	431 400	Acquired hemolytic anemia; myelofibrosis
L. W.	Absent	3	16.4	5.1	16.5	1.83	1.4	0.37	0.51	1.4	3.9	2.0	728	Acquired hemolytic anemia; L.E.D.‖
W. M.	Absent	14	13.5	11.4	33.0	2.01	0.6					2.1	537 1,361 919	Acquired hemolytic anemia; chronic lymphocytic leukemia
N. A.	Absent	7	8.4	7.1	22.0	2.35	0.6	0.46	0.36	6.4	5.5	0.6	324 432	Acquired hemolytic anemia; myelofibrosis; acute leukemia
J. L.	Absent	11		9.6	27.0	3.34	1.8	0.42	0.54	5.6	6.7	1.3	1,578 1,200	Acquired hemolytic anemia (idiopathic)
J. T.	Absent	2	32.5	8.8	26.0		1.1	0.47			9.6	2.5	542	Acquired hemolytic anemia (idiopathic)

* Mean normal value in this laboratory: Direct, 0.43 ± 0.02 (1 S.D.); incubated, 0.52 ± 0.03 (1 S.D.).
† Mean normal value in this laboratory: 1.1 ± 0.5 per cent (1 S.D.).
‡ Mean normal value in this laboratory: 4.5 ± 1.3 per cent (1 S.D.).
§ Three thousand thirty Ehrlich units per 100 Gm. stool (markedly elevated).
‖ L.E.D. = Disseminated lupus erythematosus.

anemia, Cr^{51} red cell survival studies with organ counting were performed.

Technique of Cr^{51} red cell survival studies

A. Labelling and administration of erythrocytes. Sterile technique was used throughout. Seventy-five to 90 ml. of venous blood were collected by gravity flow into a 100-ml. siliconized bottle containing 10 ml. acid-citrate-dextrose[4] solution. Seventy-five to 100 microcuries Cr^{51} (specific activity range, 0.7 to 26.0 mc. per mg.) as sodium chromate diluted in 0.85 per cent NaCl were added at once. The mixture was incubated at 37°C. for 45 minutes with frequent gentle agitation. Following incubation, 200 mg. ascorbic acid were added to prevent further uptake of Cr^{51} by red cells (21). A single washing procedure, using cold sterile saline, was done as follows: The mixture was centrifuged and the supernatant plasma was removed. The red cells were washed once with saline and finally diluted with saline to a volume approximating that of the whole blood withdrawn. These infusions were designated "washed." The washing procedure removed Cr^{51} which was not bound to erythrocytes so that more than 97 per cent of Cr^{51} infused was red-cell bound. In some instances the blood was not washed and such infusions were designated "non-washed." Cr^{51}-labelled red cells were given intravenously through an 18-gauge needle by gravity drip from the collecting bottle or in 50-ml. volumes by calibrated syringe.

B. Determination of Cr^{51} red cell survival. All samples of peripheral blood were collected in balanced oxalate (15) in approximately 6-ml. quantities. Packed cell volumes were determined. Exactly 4 ml. were pipetted into counting tubes calibrated at 4 ml. Plasma was re-

[4] Abbott Laboratories, North Chicago, Illinois. The ACD solution had the following composition per 100 ml.: Dextrose, USP 132 mg.; sodium citrate, USP 250 mg.; citric acid, USP 80 mg.

TABLE II

Donors and recipients of Cr^{51}-labelled red cells

Study No.	Donor of Cr^{51} red cells		Recipient of Cr^{51} red cells				
	Subject	Diagnosis	Subject	Diagnosis	Spleen size (*cm.* below costal margin)	Cr^{51} red-cell half-life (*days*)	Average ratio spleen Cr^{51}: liver Cr^{51}
1	D. D.	Normal	D. D.	Normal	0	34	1.6
2	D. D.	Normal	J. H.	Normal	0	38	1.8
3	T. M.	Normal	T. M.	Normal	0	42	1.1
4	M. M.	Normal	M. M.	Normal	0	30	1.3
5	D. H.	Normal	D. H.	Normal	0	32	0.9
6	D. H.	Normal	L. R.	Congenital hemolytic anemia	9	30	1.9
7	J. K.	Polycythemia	J. K.	Polycythemia	8	27	2.8
8	L. K.	Myelofibrosis	L. K.	Myelofibrosis	17	27	1.8
9	L. R.	Congenital hemolytic anemia	D. H.	Normal	0	6	3.4
10	L. R.	Congenital hemolytic anemia	L. R.	Congenital hemolytic anemia	9	10	3.4
11		Normal	E. Z.	Congenital hemolytic anemia	18	22	2.4
12	E. Z.	Congenital hemolytic anemia	E. Z.	Congenital hemolytic anemia	18	11	3.3
13	V. H.	Congenital hemolytic anemia	V. H.	Congenital hemolytic anemia	0	11	1.4
14	V. H.	Congenital hemolytic anemia	V. H.	Congenital hemolytic anemia	0	29	(post-splenectomy)
15	N. H.	Congenital hemolytic anemia	N. H.	Congenital hemolytic anemia	0	17	2.4

TABLE II—*Continued*

Study No.	Donor of Cr^{51} red cells		Recipient of Cr^{51} red cells		Spleen size (*cm.* below costal margin)	Cr^{51} red-cell half-life (*days*)	Average ratio spleen Cr^{51}: liver Cr^{51}
	Subject	Diagnosis	Subject	Diagnosis			
16		Normal	A. K.	Paroxysmal nocturnal hemoglobinuria	2	30	1.8
17	A. K.	Paroxysmal nocturnal hemoglobinuria	A. K.	Paroxysmal nocturnal hemoglobinuria	2	6	1.5
18	L. H.	Idiopathic hemolytic anemia	L. H.	Idiopathic hemolytic anemia	8	16	3.0
19		Normal	P. P.	Idiopathic hemolytic anemia	6	17	4.2
20		Normal	N. A.	Myelofibrosis	7	12	3.4
21		Normal	W. M.	Chronic lymphocytic leukemia	14	6	3.5
22		Normal	L. W.	L.E.D.*	3	19	2.0
23	J. T.	Idiopathic hemolytic anemia	J. T.	Idiopathic hemolytic anemia	2	15	1.9
24	G. S.	Myelofibrosis	G. S.	Myelofibrosis	27	22	2.0
25		Normal	J. L.	Idiopathic hemolytic anemia	6–11†	30	2.4
26	J. L.	Idiopathic hemolytic anemia	J. L.	Idiopathic hemolytic anemia	11	8	3.4
27		Normal	L. Y.	Chronic lymphocytic leukemia	10	<1	6.4

* L.E.D. = Disseminated lupus erythematosus.

† The spleen enlarged by clinical estimate from 6 cm. below costal margin to 11 cm. below costal margin during the study.

moved after centrifugation and the red cells were hemolysed by the addition of distilled water to the 4-ml. mark. Radioactivity was measured in a well-type scintillation counter.

Blood samples were drawn in most instances at frequent intervals for 24 hours following infusion. The sample showing the most radioactivity during this period was chosen the "100 per cent sample of Red Cell Cr^{51}." In some, a single 2- or 24-hour sample served as the "100 per cent sample." Subsequent samples were collected at intervals of 1 to 14 days depending upon the rate of disappearance of radioactivity from the peripheral blood. Corrections for Cr^{51} decay were obviated by counting both the "100 per cent sample" and the interval samples on the same day. Corrections for variation in plasma volumes based on hematocrit determinations were not used, and no corrections were made for the elution of Cr^{51} from red cells. The per cent survival of Cr^{51} red cells at any time after infusion was given by:

$$\frac{\text{CPM per 4-ml. interval sample}}{\text{CPM per 4-ml. "100 per cent sample"}} \times 100$$

where CPM = net radioactivity in counts per minute. Studies were usually carried to 10 per cent survival of red cell Cr^{51} except when splenectomy terminated the study sooner.

External organ counting

Organ counting was done with an uncollimated scintillation probe at the time of sample collection for Cr^{51} red cell survival. With the patient supine, the approximate centers of both anterior and lateral skin projections of the liver and lateral projection only of the spleen were estimated by physical examination. The detector was

placed on the skin at these sites so the face of the crystal was approximately parallel to the body surface. When multiple determinations of radioactivity were made from a single organ at the same time, the results were averaged. A minimum of 5,000 counts over each organ site was obtained. Radioactivity values over either organ less than 1.5 times background were not used. The ratio net spleen counts: net liver counts was determined.

Selection of subjects for Cr^{51} studies

Twenty-one subjects were studied by Cr^{51}-labelled red cell survival and organ counting techniques. Five were healthy young men without splenomegaly. Studies were made on two patients who had splenomegaly without hemolytic anemia. Radioactivity studies were made in 14 patients who had classical evidence of hemolytic anemia.

With the exception of Patient N. A., all subjects with acquired hemolytic anemia were given adrenal steroids prior to splenectomy without significant benefit. We have hesitated to advise splenectomy for acquired hemolytic anemia when the process was controlled by steroids.

RESULTS

Initial data on patients with hemolytic anemia

Pertinent clinical features and laboratory data on the 14 patients showing evidence for hemolytic anemia are presented in Table I. The final diagnosis for each subject is entered in Table I for completion, but was not made in all patients prior to labelled erythrocyte studies. All subjects but N. H. and L. H. had increased (> 280 mg. per day) fecal urobilinogen. The osmotic fragility of red cells after incubation was abnormally increased in patients with hereditary spherocytosis. Increases in mean fragility upon incubation were not seen consistently in patients with acquired hemolytic anemia. Autohemolysis after 48 hours of sterile incubation was strikingly increased in hereditary spherocytosis and in the one subject (A. K.) with paroxysmal nocturnal hemoglobinuria. In acquired hemolytic anemia the degree of autohemolysis varied within wide limits from normal to abnormal. Mechanical fragility was regularly increased only in the hereditary spherocytosis group.

Cr^{51} red cell survival and organ counting

There were 26 simultaneous determinations of Cr^{51} red cell survival and spleen Cr^{51}: liver Cr^{51} ratios in the 21 subjects. Five of these subjects received labelled red cells from a suitable donor (homologous), and at another time an infusion of their own labelled red cells (autologous). An additional survival study was done in one subject (V. H.) after splenectomy. Pertinent data on donor red cells and recipients are shown in Table II.

In Figures 1 to 9 data on Cr^{51} red cell survival and organ counting for each recipient are plotted

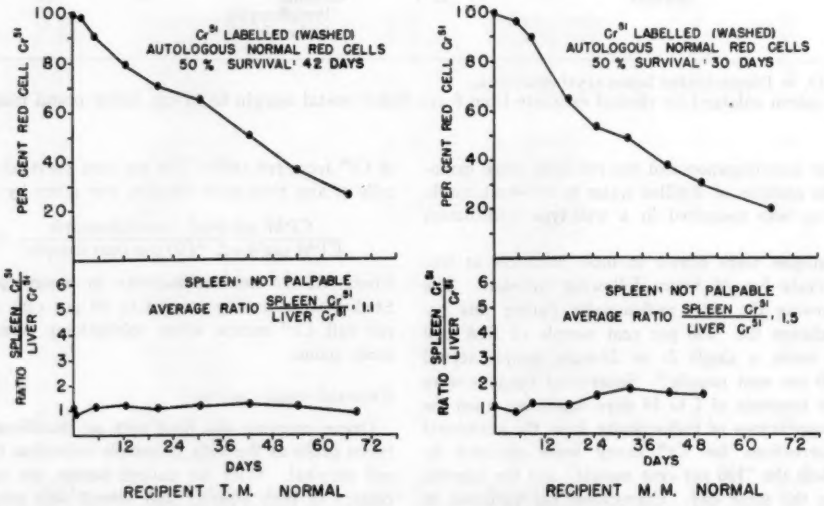

PER CENT RED CELL Cr51
Cr51 LABELLED (WASHED) AUTOLOGOUS NORMAL RED CELLS
50 % SURVIVAL: 42 DAYS
SPLEEN: NOT PALPABLE
AVERAGE RATIO SPLEEN Cr51 / LIVER Cr51: 1.1
RATIO SPLEEN Cr51 / LIVER Cr51
DAYS
RECIPIENT T. M. NORMAL
Cr51 LABELLED (WASHED) AUTOLOGOUS NORMAL RED CELLS
50 % SURVIVAL: 30 DAYS
SPLEEN: NOT PALPABLE
AVERAGE RATIO SPLEEN Cr51 / LIVER Cr51: 1.5
RECIPIENT M. M. NORMAL

FIG. 1. NORMAL RED CELLS INFUSED INTO NORMAL RECIPIENTS
Recipients T. M. and M. M. (Studies No. 3 and 4—Table II).

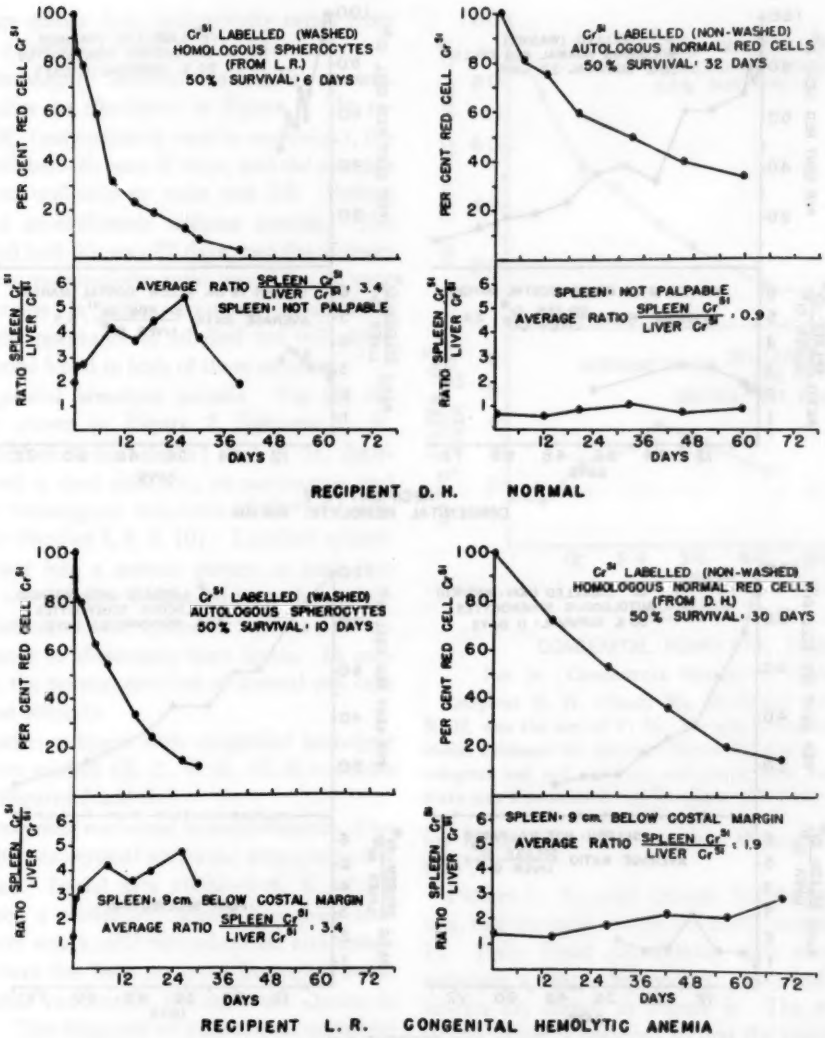
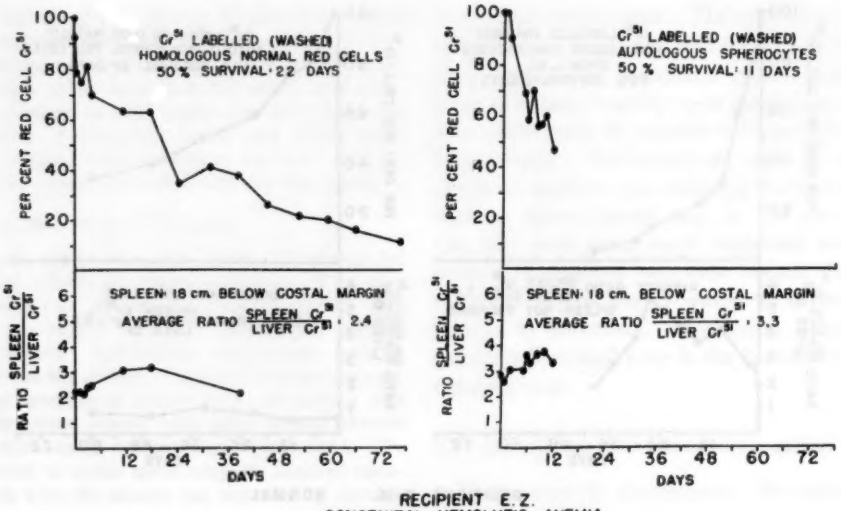

Cr51 LABELLED (WASHED) HOMOLOGOUS SPHEROCYTES (FROM L. R.) 50% SURVIVAL · 6 DAYS
Cr51 LABELLED (NON-WASHED) AUTOLOGOUS NORMAL RED CELLS 50% SURVIVAL · 32 DAYS
PER CENT RED CELL Cr51
AVERAGE RATIO SPLEEN Cr51 / LIVER Cr51 · 3.4
SPLEEN · NOT PALPABLE
AVERAGE RATIO SPLEEN Cr51 / LIVER Cr51 · 0.9
RATIO SPLEEN Cr51 / LIVER Cr51
DAYS
RECIPIENT D. H. NORMAL
Cr51 LABELLED (WASHED) AUTOLOGOUS SPHEROCYTES 50% SURVIVAL · 10 DAYS
Cr51 LABELLED (NON-WASHED) HOMOLOGOUS NORMAL RED CELLS (FROM D. H.) 50% SURVIVAL · 30 DAYS
SPLEEN · 9 cm. BELOW COSTAL MARGIN
AVERAGE RATIO SPLEEN Cr51 / LIVER Cr51 · 3.4
SPLEEN · 9 cm. BELOW COSTAL MARGIN
AVERAGE RATIO SPLEEN Cr51 / LIVER Cr51 · 1.9
RECIPIENT L. R. CONGENITAL HEMOLYTIC ANEMIA

FIG. 2. A MODEL OF RED CELL SEQUESTRATION

Recipients D. H. and L. R. (Studies No. 5, 6, 9, and 10—Table II). Patient L. R. served as a spherocyte donor in the upper and lower left studies which were carried out simultaneously. There was a rapid accumulation of splenic radioactivity as evidenced by rising spleen Cr^{51}: liver Cr^{51} ratios concomitant with a grossly reduced red cell survival in both subjects. On the right are presented the data on the infusion of Cr^{51} red cells from normal Subject D. H. into each subject simultaneously. In contrast, the red cell survival times were in the normal range and the spleen Cr^{51}: liver Cr^{51} ratios did not rise appreciably relative to changes which occurred after infusion of spherocytes.

on arithmetical coordinates. Figures 1 to 7 have the same scale to facilitate comparison of studies. The data in two subjects with severe hemolysis are shown on expanded scales in Figures 8 and 9.

A. Normal studies. Individual studies in normal subjects are depicted in Figures 1 and 2. In the five studies of normal donors and recipients, the Cr^{51} red cell half-life varied from 30 to 42 days,


Cr51 LABELLED (WASHED)
HOMOLOGOUS NORMAL RED CELLS
50 % SURVIVAL: 22 DAYS
PER CENT RED CELL Cr51
SPLEEN: 18 cm. BELOW COSTAL MARGIN
AVERAGE RATIO SPLEEN Cr51 / LIVER Cr51 : 2.4
RATIO SPLEEN Cr51 / LIVER Cr51
DAYS
Cr51 LABELLED (WASHED)
AUTOLOGOUS SPHEROCYTES
50 % SURVIVAL: 11 DAYS
SPLEEN: 18 cm. BELOW COSTAL MARGIN
AVERAGE RATIO SPLEEN Cr51 / LIVER Cr51 : 3.3
RECIPIENT E. Z.
CONGENITAL HEMOLYTIC ANEMIA

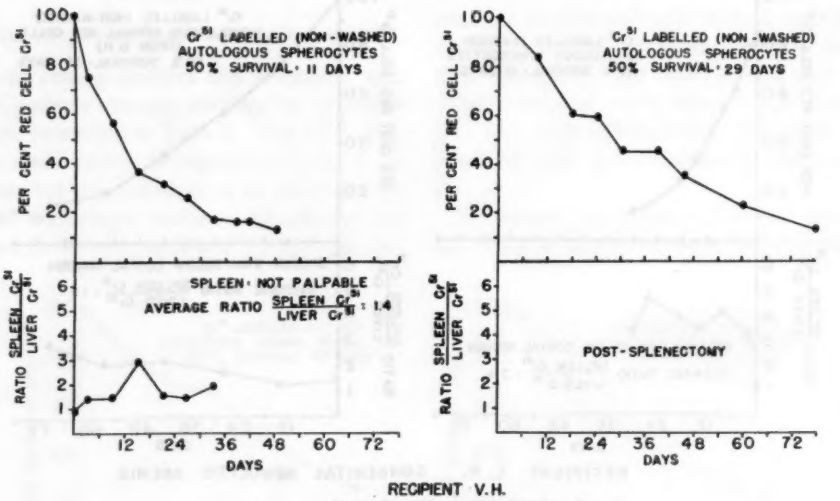


FIG. 3. CONGENITAL HEMOLYTIC ANEMIA

Recipient E. Z. (Studies No. 11 and 12—Table II). Homologous normal and autologous (spherocyte) studies are shown in the upper plots. The homologous normal red cell data is shown on the left. The patient suffered an infection between the 18th and 25th days of this study coincident with a fall in hematocrit. The drop in the red cell survival curve at this time produced a red cell half-life slightly less than normal (22 days). After recovery the curve became normal. On the right is shown the autologous (spherocyte) plot. The red cell half-life was grossly reduced concomitant with a rapid accumulation of splenic radioactivity. This study was terminated by splenectomy on the 12th day after infusion.

Recipient V. H. (Studies No. 13 and 14—Table II). Autologous studies before and after splenectomy are shown in the lower plots. The pre-splenectomy study plotted on the left demonstrates only a slight early rise in the spleen: liver radioactivity ratio. The average ratio was not high. The post-splenectomy survival of red cells (spherocytes) shown on the right was normal. The spleen weighed 200 Gm.

and average spleen : liver radioactivity ratios from 0.9 to 1.8 (Table II—Studies 1 to 5).

B. Splenomegaly without hemolytic anemia. These studies are illustrated in Figure 4. In recipient J. K. (polycythemia vera in remission), the Cr^{51} red cell half-life was 27 days, and the average spleen : liver radioactivity ratio was 2.8. Patient L. K. had myelofibrosis without anemia. The Cr^{51} red cell half-life was 27 days, and the average spleen : liver radioactivity ratio was 1.8. A very slight increase in ratios occurred concomitant with the disappearance of labelled red cells from the peripheral blood in both of these subjects.

C. Congenital hemolytic anemia. For the observations shown in Figure 2, Subjects L. R. (congenital hemolytic anemia) and D. H. (normal) served a dual role, *i.e.*, as autologous and reciprocal homologous recipients of Cr^{51} red cells (Table II—Studies 5, 6, 9, 10). Labelled spherocytes infused into a normal person or back into the donor were rapidly eliminated from the peripheral circulation, and the spleen Cr^{51} : liver Cr^{51} ratios increased to abnormally high levels. In contrast were the normal survival of normal red cells in the same subjects.

Three other subjects with congenital hemolytic anemia were studied (E. Z., V. H., N. H.—Tables I and II, Figures 3 and 3a).

D. Paroxysmal nocturnal hemoglobinuria. One patient with paroxysmal nocturnal hemoglobinuria was studied. Initial data (Subject A. K., Table I) indicated a moderately severe hemolytic anemia. There was a mild thrombopenia and leukopenia. There has been no gross hemoglobinuria. Studies with radioactive red cells are shown in Figure 5. The diagnosis of paroxysmal nocturnal hemoglobinuria was not considered until a normal red cell survival was obtained after the infusion of labelled cells from a normal donor. Survival of the patient's own labelled red cells was markedly reduced suggesting an intracorpuscular defect. Previous data did not suggest hereditary spherocytosis (Table I). The spleen Cr^{51} : liver Cr^{51} ratios did not indicate selective splenic sequestration of red cells in either study. The acid hemolysis test of Ham (15) was positive.

E. Acquired hemolytic anemia. Nine patients with acquired hemolytic anemia were studied with radioactive red cell techniques (Table II—Studies 18 to 27). The data are shown in Figures 6 to 9.

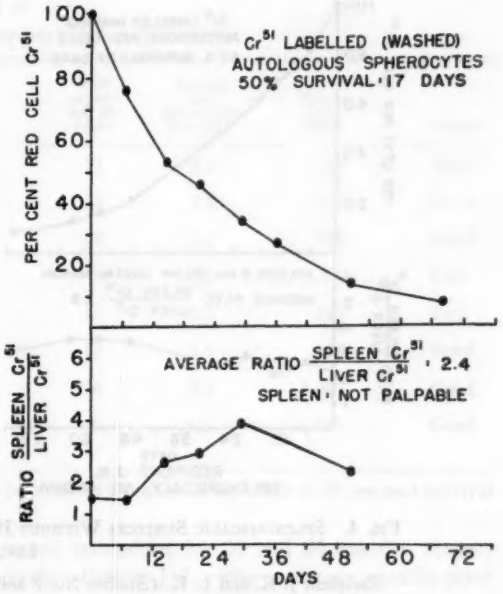


FIG. 3a. CONGENITAL HEMOLYTIC ANEMIA

Recipient N. H. (Study No. 15—Table II). Patient N. H. was the son of V. H. He was asymptomatic with initial evidence for minimal hemolytic anemia. The autologous red cell survival was moderately reduced and there was a prominent rise in spleen Cr^{51} : liver Cr^{51} ratios.

Patient L. Y., with chronic lymphocytic leukemia, had the most severe hemolytic anemia (Table I). Daily blood transfusions were necessary to maintain a stable hematocrit. Labelled red cell studies are shown in Figure 9. The scale used in this graph is enlarged so that the rapid changes may be clearly seen. The 50 per cent survival of Cr^{51}-labelled red cells from a normal donor was less than one day. With the rapid decline in the peripheral blood radioactivity there was an approximately reciprocal rise in the spleen Cr^{51} : liver Cr^{51} ratio. The average ratio of spleen to liver radioactivity was 6.4.

Splenectomy

The effect of splenectomy upon the hemolytic process in eight patients is given in Table III. The two patients with congenital hemolytic anemia, V. H. and E. Z., had the anticipated good response.

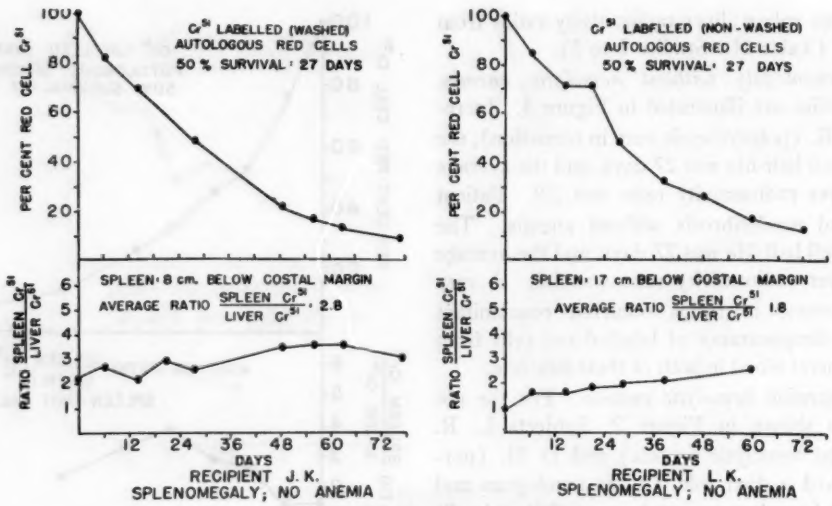

Cr51 LABELLED (WASHED) AUTOLOGOUS RED CELLS 50 % SURVIVAL · 27 DAYS
PER CENT RED CELL Cr51
SPLEEN · 8 cm. BELOW COSTAL MARGIN
AVERAGE RATIO SPLEEN Cr51 / LIVER Cr51 · 2.8
RATIO SPLEEN Cr51 / LIVER Cr51
DAYS
RECIPIENT J. K.
SPLENOMEGALY; NO ANEMIA
Cr51 LABELLED (NON-WASHED) AUTOLOGOUS RED CELLS 50 % SURVIVAL · 27 DAYS
SPLEEN · 17 cm. BELOW COSTAL MARGIN
AVERAGE RATIO SPLEEN Cr51 / LIVER Cr51 1.8
RECIPIENT L. K.
SPLENOMEGALY; NO ANEMIA

FIG. 4. SPLENOMEGALIC SUBJECTS WITHOUT HEMOLYTIC ANEMIA: AUTOLOGOUS Cr^{51} RED CELL INFUSIONS

Recipient J. K. and L. K. (Studies No. 7 and 8—Table II). These studies serve to illustrate the effect of splenomegaly without hemolytic anemia on the spleen Cr^{51}: liver Cr^{51} ratios. Subject J. K. had polycythemia vera in remission, and L. K. had myelofibrosis.

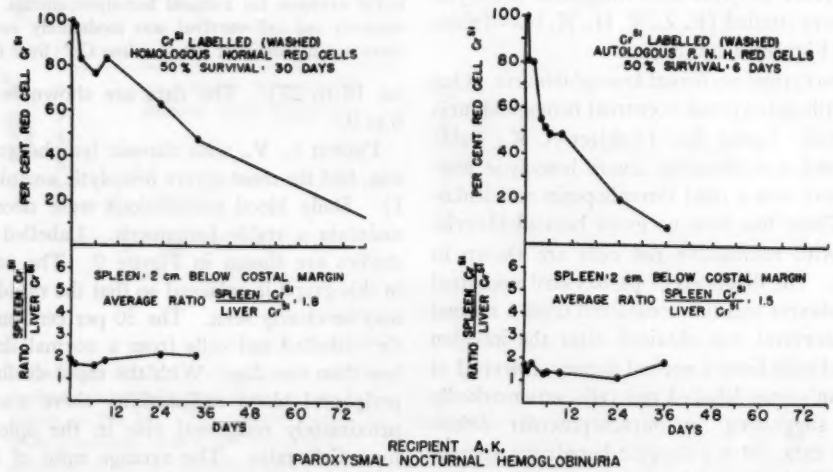

Cr51 LABELLED (WASHED) HOMOLOGOUS NORMAL RED CELLS 50 % SURVIVAL · 30 DAYS
PER CENT RED CELL Cr51
SPLEEN · 2 cm. BELOW COSTAL MARGIN
AVERAGE RATIO SPLEEN Cr51 / LIVER Cr51 · 1.8
RATIO SPLEEN Cr51 / LIVER Cr51
DAYS
Cr51 LABELLED (WASHED) AUTOLOGOUS P. N. H. RED CELLS 50 % SURVIVAL · 6 DAYS
SPLEEN · 2 cm. BELOW COSTAL MARGIN
AVERAGE RATIO SPLEEN Cr51 / LIVER Cr51 · 1.5
RECIPIENT A. K.
PAROXYSMAL NOCTURNAL HEMOGLOBINURIA

FIG. 5. PAROXYSMAL NOCTURNAL HEMOGLOBINURIA: HOMOLOGOUS NORMAL AND AUTOLOGOUS Cr^{51} RED CELL INFUSIONS

Recipient A. K. (Studies No. 16 and 17—Table II). The homologous infusion data are shown on the left, and the survival of these cells was normal. On the right, the autologous infusion data are given. Note the rapid rate of destruction of the patient's own labelled cells suggesting an intracorpuscular defect. There was no selective splenic uptake of Cr^{51} in either study as determined by spleen Cr^{51}: liver Cr^{51} ratios.

TABLE III

Effect of splenectomy in eight patients with hemolytic anemia

Patient	Diagnosis	Cr^{51}-labelled red-cell infusion	Cr^{51} red-cell half-life (*days*)	Average ratio spleen Cr^{51}: liver Cr^{51}	Spleen wt. (*Gm.*)	Result
V. H.	Congenital hemolytic anemia	Autologous	11	1.4	200	Good
E. Z.*	Congenital hemolytic anemia	Autologous	11	3.3	1,000	Good
L. H.*	Idiopathic hemolytic anemia	Autologous	16	3.0	958	Good
J. L.*	Idiopathic hemolytic anemia	Autologous	9	3.3	1,380	Fair
J. T.	Idiopathic hemolytic anemia	Autologous	15	1.9	323	Poor
N. A.	Myelofibrosis	Homologous normal	12	3.4	518	Good
W. M.	Chronic lymphocytic leukemia	Homologous normal	6	3.5	1,860	Good
L. Y.	Chronic lymphocytic leukemia	Homologous normal	<1	6.4	1,045	Good

* Cr^{51}-labelled red cell survival with organ counting was interrupted by splenectomy at 40 to 50 per cent survival of labelled erythrocytes.

Patient V. H. (Table II—Study 14, and Figure 3) had a normal autologous Cr^{51} red cell survival after splenectomy.

Each of the five patients with acquired hemolytic anemia benefitting from splenectomy had an average spleen: liver radioactivity ratio of 3.0 or higher following the infusion of Cr^{51}-labelled red cells. Probably more important than the average ratio is evidence for a rising ratio of spleen: liver radioactivity, suggesting selective splenic trapping of red cells. Data from Patients N. A. (Figure 6), J. L. (Figure 8), and L. Y. (Figure 9) show striking evidence of increasing splenic radioactivity as compared to the liver during the period of rapid removal of labelled red cells from the circulation. Studies on W. M. (Figure 6) show a high initial spleen: liver radioactivity ratio, but this point was obtained 12 hours after the infusion of cells and may not be representative of earlier splenic radioactivity. The data from Patient L. H. (Figure 6) are suggestive of splenic accumulation of trapped red cells, but we lack data for the first several days of the study. In contrast, Patient J. T. (Figure 7) had an average ratio of 1.9 and was not improved by splenectomy. There was no evidence of a rising spleen: liver radioactivity ratio in this patient.

The results given in Table III represent the effect of splenectomy upon the hemolytic anemia and not the primary disease. Following splenectomy, Patients L. H. and W. M. have had sustained remissions for 20 and 30 months, respectively. Patient J. L., who in the six months prior to splenectomy required at least 19 blood transfusions, did not require transfusions for three months post-operatively. He expired at home four and one-half months following splenectomy. A primary diagnosis was not established. Patient N. A., who had developed myelofibrosis and hemolytic anemia following polycythemia vera, received dramatic relief of anemia for at least 12 months post-splenectomy. Fourteen months after splenectomy this patient had developed leukemia without evidence of hemolytic anemia. He died two months later in the hospital with severe anemia and blasts of undetermined type in the peripheral blood and bone marrow. Patient L. Y. (chronic lymphatic leukemia), whose hemolysis was so severe as to require daily blood transfusions, was dramatically relieved by splenectomy. There was no evidence for hemolytic anemia when this patient was seen in the hospital a few days prior to his death three and one-half months post-operatively.

DISCUSSION

The technique of red cell labelling and estimating erythrocyte survival with radioactive chromium used in this study is similar to that employed by Ebaugh, Emerson and Ross (22). Red cell survival data reported here are based upon the radioactivity in red cells per unit volume of whole blood

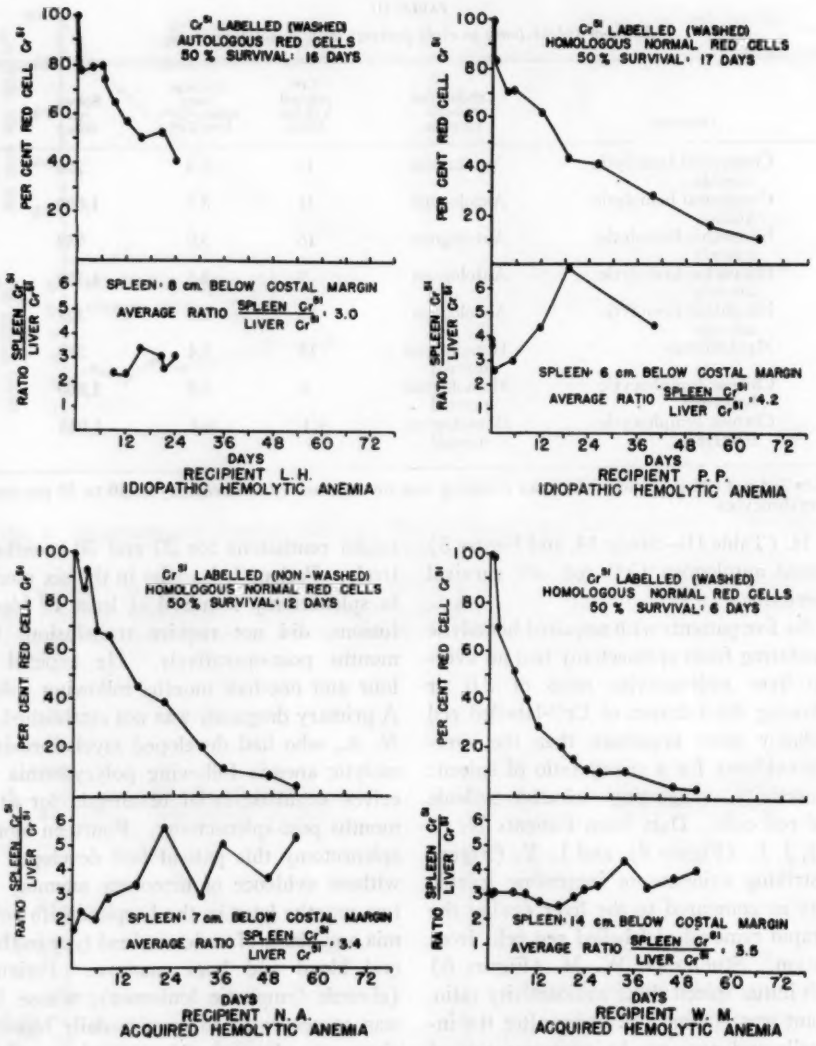

Cr51 LABELLED (WASHED)
AUTOLOGOUS RED CELLS
50 % SURVIVAL: 16 DAYS
PER CENT RED CELL Cr51
SPLEEN: 8 cm. BELOW COSTAL MARGIN
AVERAGE RATIO SPLEEN Cr51 / LIVER Cr51 = 3.0
RATIO SPLEEN Cr51 / LIVER Cr51
DAYS
RECIPIENT L.H.
IDIOPATHIC HEMOLYTIC ANEMIA
Cr51 LABELLED (WASHED)
HOMOLOGOUS NORMAL RED CELLS
50 % SURVIVAL: 17 DAYS
SPLEEN: 6 cm. BELOW COSTAL MARGIN
AVERAGE RATIO SPLEEN Cr51 / LIVER Cr51 = 4.2
RECIPIENT P.P.
IDIOPATHIC HEMOLYTIC ANEMIA
Cr51 LABELLED (NON-WASHED)
HOMOLOGOUS NORMAL RED CELLS
50 % SURVIVAL: 12 DAYS
SPLEEN: 7 cm. BELOW COSTAL MARGIN
AVERAGE RATIO SPLEEN Cr51 / LIVER Cr51 = 3.4
RECIPIENT N.A.
ACQUIRED HEMOLYTIC ANEMIA
Cr51 LABELLED (WASHED)
HOMOLOGOUS NORMAL RED CELLS
50 % SURVIVAL: 6 DAYS
SPLEEN: 14 cm. BELOW COSTAL MARGIN
AVERAGE RATIO SPLEEN Cr51 / LIVER Cr51 = 3.5
RECIPIENT W.M.
ACQUIRED HEMOLYTIC ANEMIA

FIG. 6. ACQUIRED HEMOLYTIC ANEMIA

Recipient L. H. (Study No. 18—Table II). Patient L. H. had splenomegaly, thrombopenic purpura and mild hemolytic anemia of unknown cause. Autologous infusion data are given in the upper left graph. The patient's own labelled cells were eliminated at a moderately increased rate (50 per cent survival in 16 days). The average spleen Cr^{51}: liver Cr^{51} ratio was 3.0. This study was terminated by splenectomy on the 25th day after infusion. There was a good response to splenectomy.

Recipient P. P. (Study No. 19—Table II). On the upper right homologous infusion data for Patient P. P. are shown. The patient had minimal anemia. The survival of homologous Cr^{51}-labelled red cells substantiated a moderate hemolytic process (50 per cent survival in 17 days). The rise in spleen Cr^{51}: liver Cr^{51} ratios, however, is striking and approximates in a reciprocal manner the fall in peripheral blood red cell Cr^{51}. The average spleen Cr^{51}: liver Cr^{51} ratio was 4.2. Splenectomy was not performed.

Recipient N. A. (Study No. 20—Table II). This patient had myelofibrosis and acquired hemolytic anemia following polycythemia vera. He was given Cr^{51}-labelled red cells from a

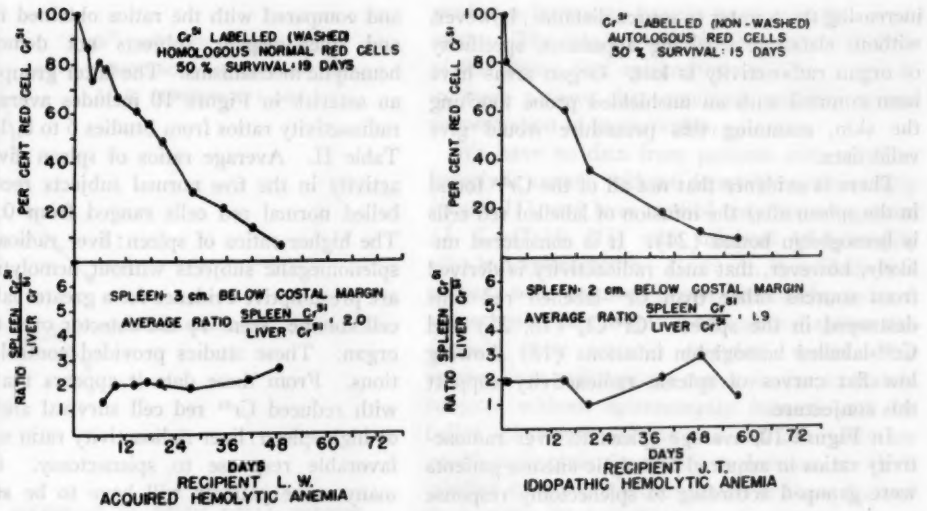

Cr51 LABELLED (WASHED) HOMOLOGOUS NORMAL RED CELLS 50 % SURVIVAL: 19 DAYS
PER CENT RED CELL Cr51
SPLEEN: 3 cm. BELOW COSTAL MARGIN
AVERAGE RATIO SPLEEN Cr51 / LIVER Cr51 = 2.0
RATIO SPLEEN Cr51 / LIVER Cr51
DAYS
RECIPIENT L. W.
ACQUIRED HEMOLYTIC ANEMIA
Cr51 LABELLED (NON-WASHED) AUTOLOGOUS RED CELLS 50 % SURVIVAL: 15 DAYS
SPLEEN: 2 cm. BELOW COSTAL MARGIN
AVERAGE RATIO SPLEEN Cr51 / LIVER Cr51 = 1.9
RECIPIENT J. T.
IDIOPATHIC HEMOLYTIC ANEMIA

FIG. 7. ACQUIRED HEMOLYTIC ANEMIA

Recipient L. W. (Study No. 22—Table II). Patient L. W. had a positive "LE test." The labelled red cell data in this subject are shown on the left. The survival of normal donor's cells was moderately reduced (half-life, 19 days) and there was no evidence for selective sequestration of labelled erythrocytes in the spleen by external counting (average spleen Cr^{51}: liver Cr^{51} ratio, 2.0). Splenectomy was not done.

Recipient J. T. (Study No. 23—Table II). Patient J. T. received his own labelled red cells and is illustrated on the right. The red cell survival was reduced (half-life, 15 days), and there was no indication from spleen Cr^{51}: liver Cr^{51} ratios for splenic sequestration of labelled erythrocytes. This patient did not benefit from splenectomy.

which assumes a constant whole blood volume. It is believed this method gives a better estimation of Cr^{51} red cell survival than techniques based on measurements of radioactivity per unit volume of packed cells. The latter method assumes a constant red cell volume, an assumption which may not be correct in hemolytic states (23).

There are many errors involved in the estimation of relative organ radioactivity by external monitoring of gamma emissions. Variations in counter positioning, counter-to-source distance, organ size, and radioactivity in adjacent viscera all affect the results. We have estimated the anatomic center of the surface projection of the liver and spleen by percussion, and, by using external landmarks, repositioned the probe at the same area in subsequent observations. Particular care must be employed in positioning the detector over the smaller organ (*e.g.*, small spleen). If the approximate center is not located or there are moderate variations in day-to-day positioning, relatively large errors may be made in evaluating organ radioactivity. There are geometric limits of radioactivity accepted by the scintillator at close range. Consequently, less radioactivity is detectable over the large organ as compared to the small one, even though both contain equal quantities of isotope. This problem can theoretically be solved by

normal donor. The data are shown in the lower left graph. There was a rapidly rising spleen Cr^{51}: liver Cr^{51} ratio concomitant with a rapid rate of Cr^{51} red cell removal from the peripheral blood. This patient responded well to splenectomy but 14 months later the clinical features of acute leukemia led to his demise.

Recipient W. M. (Study No. 21—Table II). This patient had chronic lymphatic leukemia with an acquired hemolytic anemia. The survival of Cr^{51}-labelled red cells from a normal donor was grossly reduced (half-life, 6 days). There was an associated rise in spleen Cr^{51}: liver Cr^{51} ratio (average ratio, 3.5). This patient had an excellent response to splenectomy. He has had no treatment or anemia during the two and one-half years since.

increasing the counter to source distance; however, without elaborate shielding apparatus, specificity of organ radioactivity is lost. Organ areas have been counted with an unshielded probe touching the skin, assuming this procedure would give valid data.

There is evidence that not all of the Cr^{51} found in the spleen after the infusion of labelled red cells is hemoglobin bound (24). It is considered unlikely, however, that such radioactivity is derived from sources other than Cr^{51}-labelled red cells destroyed in the spleen. $Cr^{51}Cl_3$ (13, 25) and Cr^{51}-labelled hemoglobin infusions (13) showing low flat curves of splenic radioactivity support this conjecture.

In Figure 10, average spleen to liver radioactivity ratios in acquired hemolytic anemia patients were grouped according to splenectomy response and compared with the ratios obtained in normal and splenomegalic subjects not demonstrating hemolytic mechanisms. The latter group noted by an asterisk in Figure 10 includes average organ radioactivity ratios from Studies 6 to 8, 11 and 25, Table II. Average ratios of spleen: liver radioactivity in the five normal subjects receiving labelled normal red cells ranged from 0.9 to 1.8. The higher ratios of spleen: liver radioactivity in splenomegalic subjects without hemolytic anemia are presumptive evidence for a greater labelled red cell volume "seen" by the detector over the larger organ. These studies provided control observations. From these data it appears that patients with reduced Cr^{51} red cell survival and a rising or high spleen: liver radioactivity ratio will have a favorable response to splenectomy. Obviously many more patients will have to be studied by

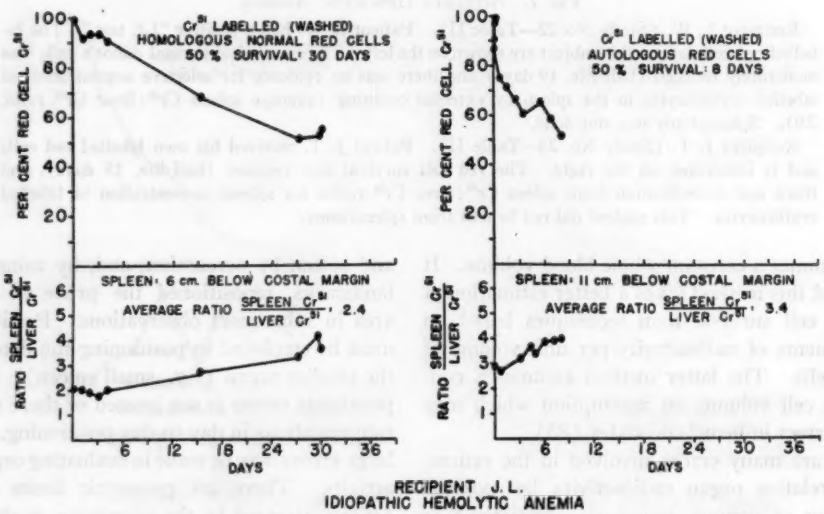

Cr51 LABELLED (WASHED)
HOMOLOGOUS NORMAL RED CELLS
50 % SURVIVAL: 30 DAYS
PER CENT RED CELL Cr51
SPLEEN: 6 cm. BELOW COSTAL MARGIN
AVERAGE RATIO SPLEEN Cr51 / LIVER Cr51: 2.4
RATIO SPLEEN Cr51 / LIVER Cr51
DAYS
Cr51 LABELLED (WASHED)
AUTOLOGOUS RED CELLS
50 % SURVIVAL: 8 DAYS
SPLEEN: 11 cm. BELOW COSTAL MARGIN
AVERAGE RATIO SPLEEN Cr51 / LIVER Cr51: 3.4
RECIPIENT J. L.
IDIOPATHIC HEMOLYTIC ANEMIA

FIG. 8. ACQUIRED HEMOLYTIC ANEMIA

(Note change in scale of abscissa.)

Recipient J. L. (Studies No. 25 and 26—Table II). Patient J. L. had severe hemolytic disease. The survival of compatible labelled donor red cells ("homologous"), given on the left, was normal. During this study the spleen increased in size from 6 cm. below costal margin to 11 cm. below costal margin. The average spleen Cr^{51}: liver Cr^{51} ratio was 2.4 prior to interruption of the study at 30 days for blood transfusions.

The autologous study given on the right showed a grossly reduced red cell half-life of the patient's cells, and concomitantly a rapid selective accumulation of radioactivity in the spleen. The spleen Cr^{51}: liver Cr^{51} ratios averaged 3.4 when the study was terminated by splenectomy on the ninth day.

Subsequent detailed study of the Rh genotype of the patient and compatible donor revealed a difference. The patient was found to be CDE/CDe and the donor CDe/Cde. These findings are compatible with, though not diagnostic of, an anti-Rh type specific hemolytic anemia. The acid hemolysis test of Ham was negative.

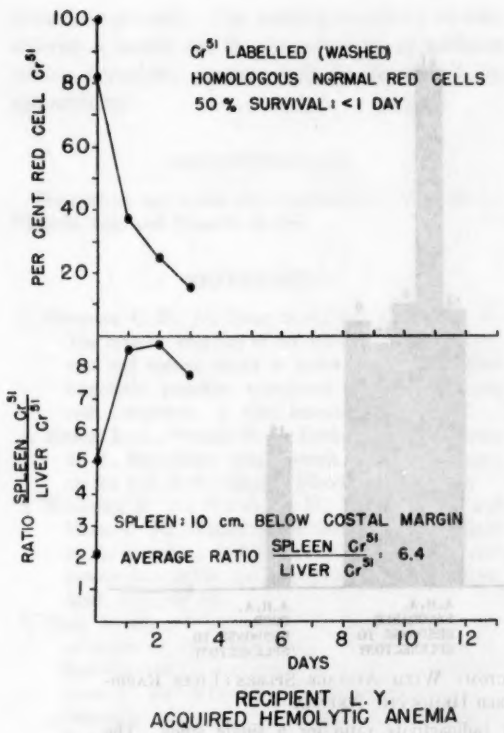

PER CENT RED CELL Cr51
Cr51 LABELLED (WASHED)
HOMOLOGOUS NORMAL RED CELLS
50 % SURVIVAL: <1 DAY
RATIO SPLEEN Cr51 / LIVER Cr51
SPLEEN: 10 cm. BELOW COSTAL MARGIN
AVERAGE RATIO SPLEEN Cr51 / LIVER Cr51 : 6.4
DAYS
RECIPIENT L. Y.
ACQUIRED HEMOLYTIC ANEMIA

FIG. 9. ACQUIRED HEMOLYTIC ANEMIA

(Note change in scale of abscissa.)

Recipient L. Y. (Study No. 27—Table II). Patient L. Y. had chronic lymphatic leukemia with severe hemolytic anemia. The accumulation of radioactivity in the spleen is striking and the plot of spleen Cr^{51}: liver Cr^{51} ratios is approximately reciprocal to the plot of disappearance of Cr^{51}-labelled erythrocytes from the peripheral blood. The average spleen Cr^{51}: liver Cr^{51} ratio was 6.4. This patient had a good response to splenectomy.

these techniques before one can say that they provide valid criteria for preoperative prediction of the results of splenectomy. In a correlation of splenic sequestration with results of splenectomy the following combinations are possible:

	Evidence for sequestration	Benefit from splenectomy
1)	+	+
2)	+	−
3)	−	+
4)	−	−

Our data from patients with acquired hemolytic anemia show five examples of the first combination and one example of the fourth. We have not observed No. 2 or 3. Thus it cannot be claimed that the data provide evidence enabling one to predict a failure to benefit from removal of the spleen. The data are considered evidence in favor of the theory that splenectomy will be beneficial in those patients demonstrating selective splenic sequestration of erythrocytes.

We have no data from patients with acquired hemolytic anemia without detectable splenomegaly (Table II), and have studied only the one patient (J. T.—Table III) with minimal splenomegaly who had splenectomy. However, it is likely that patients with acquired hemolytic anemia, red cell sequestration in the spleen and normal spleen size (should these features co-exist) would show spleen: liver radioactivity ratios similar to that in subjects without splenomegaly infused with labelled spherocytes (Figures 2 and 3a). We are unable to explain the failure to demonstrate Cr^{51}-labelled spherocyte accumulation in the spleen of the patient with hemolytic anemia without splenomegaly (V. H.—Figure 3).

Cr^{51}-labelled erythrocyte studies, in addition to the estimation of organ accumulation of red cells, may occasionally suggest mechanisms of hemolytic disease previously unsuspected. Clinical evaluation of Patient A. K. had indicated a brisk hemolytic anemia, mild thrombopenia and leukopenia. Splenectomy might have been advised for "hypersplenism" had not erythrocyte survival studies indicated an intraco puscular defect (Figure 5). There was no evidence for congenital hemolytic anemia (Table I), and the acid hemolysis test was unequivocally positive establishing the diagnosis of paroxysmal nocturnal hemoglobinuria. Organ radioactivity ratios in this patient did not suggest splenic accumulation of autologous or homologous erythrocytes. These studies are in accord with the usual observation that patients with this disorder are not helped by splenectomy (18). On the other hand, Jandl and his colleagues have reported that splenectomy was beneficial in a patient with this disorder in whom splenic sequestration of autologous Cr^{51}-labelled red cells had been demonstrated (13).

Studies with radioactive red cells in Patient J. L. were of particular interest. This patient had severe hemolytic anemia of unknown type. The survival of "homologous" normal Cr^{51}-labelled red cells was normal. The relatively marked rise of the spleen: liver radioactivity ratio during this study (Figure 8) was considered secondary to an

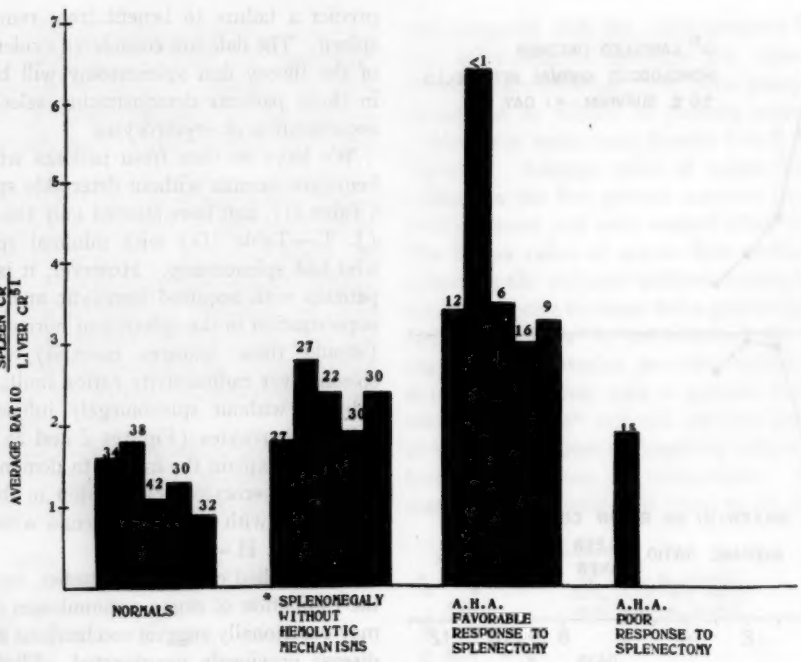


FIG. 10. CORRELATION OF RESPONSE TO SPLENECTOMY WITH AVERAGE SPLEEN: LIVER RADIOACTIVITY RATIOS IN ACQUIRED HEMOLYTIC ANEMIA

Each bar represents the average spleen: liver radioactivity ratio for a single study. The number at the top of each bar represents the Cr^{51} red cell half-life in days.
A.H.A. = Acquired hemolytic anemia.
* See text.

expanding spleen red cell volume in an enlarging spleen. An even greater accumulation of splenic radioactivity with a grossly reduced red cell survival occurred after infusion of the patient's own labelled red cells. A negative family history and conventional laboratory data excluded congenital hemolytic anemia (Table I). The acid hemolysis test for paroxysmal nocturnal hemoglobinuria was negative. Further typing of the donor's and the patient's red cells, however, revealed a difference in Rh genotypes. We were unable to repeat a study using truly homologous erythrocytes, but the observations made suggested an Rh type-specific hemolytic anemia.

Prior to labelled red cell studies, our laboratory data in acquired hemolytic anemia did not provide criteria for predicting response to splenectomy. This observation is in accord with that recently reported by Chertkow and Dacie (26). Gross splenomegaly, however, was common to all patients with acquired hemolytic anemia showing evidence for splenic accumulation of Cr^{51}-labelled red cells. Patients with acquired hemolytic anemia and splenic sequestration of labelled red cells did not have spherocytosis in the peripheral blood as judged by osmotic fragility studies.

SUMMARY

The ratio spleen: liver radioactivity as determined by external scintillation counting after the infusion of Cr^{51}-labelled erythrocytes has been used to determine organ localization of red cells in seven control subjects and 14 patients with hemolytic anemia. As anticipated, the spleen accumulated radioactivity rapidly following the transfusion of labelled spherocytes. Some patients with acquired hemolytic anemia showed gross evidence of trapping of radioactive red cells in their spleen. Removal of the spleen in five such patients was beneficial in each. One patient who showed no evidence for splenic trapping of red cells was splenectomized without beneficial effects on the

hemolytic process. The method described is considered a useful aid in the selection of patients whose hemolytic process will be benefitted by splenectomy.

ACKNOWLEDGMENT

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THE THYROID HORMONE–PLASMA PROTEIN COMPLEX IN MAN. I. DIFFERENCES IN DIFFERENT STATES OF THYROID FUNCTION [1, 2]

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The present consensus characterizes thyrotoxicosis,[4] a disease of unknown etiology, by the quantitative overproduction and release by the thyroid of excessive amounts of normal thyroid hormone (1–4). According to this concept, the distinctive abnormal signs, symptoms, and laboratory findings of hypermetabolism in patients with diffuse toxic goiter are attributed to the effects of increased amounts of the normal hormone upon peripheral tissue metabolism. Plummer (5), among others, proposed an alternate theory, based chiefly upon clinical observations, which ascribed various manifestations of the disease to the effects of 1) an excess of normal hormone, and 2) a *qualitatively* abnormal hormone, which he suggested was abnormal because of incomplete iodination. In contrast, he suggested that the abnormality in patients with toxic *nodular* goiter was due solely to an excess of normal thyroid hormone. Salter and Johnston (6) concluded, on the basis of acetone precipitation of acidified sera, that the plasma thyroid hormone in hyperthyroidism differed from thyroxin added *in vitro* to normal and hypothyroid sera to levels of protein-bound iodine comparable to those observed in hyperthyroidism. Means (1), however, found no biochemical evidence to support Plummer's two-product theory of dysthyroidism. Recent studies (2, 7–10), using more precise isotopic and chromatographic methods, have, moreover, failed to indicate any qualitative difference in the hormone circulating in the hyperthyroid as compared to the euthyroid state.

This report deals with three series of experiments employing biological test systems (infusion into dogs and *in vitro* rat diaphragm "uptake" studies) to investigate the question of the qualitative identity of the thyroid hormone-plasma protein complex in euthyroid and hyperthyroid states in man. The consistent differences observed are not adequately explained by present concepts and indicate 1) a qualitative difference in the nature of the hormone-protein complex in the hyperthyroid as compared to the euthyroid states, or 2) a differential effect of a plasma factor(s) on the disappearance from the plasma or tissue incorporation of the hormone in these two states.

EXPERIMENTAL METHODS AND RESULTS

Series 1—Infusion of endogenously I-131-labelled human thyroid hormone—Rate of disappearance in dog

A. *Methods.* Data identifying the donors and the plasma infused are presented in Table I. Eight euthyroid patients (two with thyroid carcinoma and six with angina pectoris or congestive failure), and ten with typical diffuse toxic goiter served as donors. The euthyroid subjects received for therapeutic purposes 10.2 to 61.9 mc. I-131, which delivered an estimated thyroid radiation of 1,610 to 55,000 R.E.P. (11, 12). The thyrotoxic patients received 4.7 to 15.4 mc. I-131, or 8,500 to 15,300 R.E.P. to their thyroid glands.

[1] This investigation was supported by research grants (A-140) from the National Institute of Arthritis and Metabolic Diseases of the National Institutes of Health, United States Public Health Service.

[2] Portions of this material have been presented at the Annual Meeting of the American Goiter Association, 1954, and the Annual Meeting of the Society for Clinical Investigation, 1955.

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[4] For this discussion, we refer to a disease associated with increased activity of the autonomic nervous system, characterized by diffuse enlargement of the thyroid gland, eye signs, tachycardia, tremor, hypermetabolism. It includes exophthalmic goiter, hyperthyroidism, Graves', Basedow's, or Parry's disease associated with diffuse toxic goiter.

TABLE I

Infusion of endogenously I-131-labelled human plasma into dogs

Experiment No.	Donor: Thyroid status*	Donor: Dose	Donor: 24-hr. I-131-thyroid uptake (11)	Donor: Total R.E.P. to thyroid gland (11, 12) ($\times 10^3$)	Donor: R.E.P. β-2nd 24 hrs. (16) ($\times 10^3$)	Infusion: Interval between Rx and infusion	Infusion: Volume infused	Infusion: Total counts infused ($\times 10^3$)	Infusion: PBI-131 $\times$100 Total I-131 (14)	Infusion: But. I-131 $\times$100 PBI-131	Recipient: Weight	Recipient: Sex	Recipient: Hours followed	Results: Half-time of disappearance	
		(*mc.*)	(%)			(*days*)	(*ml.*)		(%)	(%)	(*Kg.*)			(*hrs.*)	
1	Eu. Ca.	61.9	28.6	55.0	4.7	10	30	559	94.5		10.7	M	56	27.0	
2	Eu. Ca.	40.9	38.6	42.0	3.1	5	35	930	89.4		5.9	F	30	21.0	
3	Eu. Card.	14.6	40.5	34.0	2.0	6	50	150	58.6	96.1	12.7	F	24	34.2	
4	Eu. Card.	22.0	24.2	29.0	2.2	6	45	332	51.0	76.0	10.2	M	24	23.0	
5	Eu. Card.	15.4	36.4	31.0	2.3	6	50	115	53.8	88.0	13.6	M	46	32.0	
6	Eu. Card.	11.1	24.1	28.0	1.0	5	60	44	82.0	88.0	11.4	M	52	33.8	
7	Eu. Card.	10.2	44.0	22.0	1.8	6	52	170	75.0	92.0	12.4	M	96	37.6	
8	Eu. Card.	11.6	13.2	1.6	0.3	6	35	123	64.5	75.0	6.0	M	45	27.0	Av. $=29.5 \pm 5.5$
9	Tx	6.6	81.6	9.6	1.1	3	30	242	82.0	88.8	12.7	M	46	12.0	
10	Tx	4.7	77.6	10.0	1.1	5	21	187	95.0		8.6	F	28	12.6	
11	Tx	10.8	90.6	12.0	1.0	6	35	966	97.5		6.0	F	24	12.4	
12	Tx	7.7	76.5	8.5	0.8	4	40	656	94.6	83.8	13.6	M	51	10.2	
13	Tx	9.5	72.6	11.9	1.3	5	50	2,016	90.0	92.0	13.6	M	41	12.0	
14	Tx	6.2	59.7	12.4	1.4	5	60	836	100.0	100.0	10.0	F	24	12.0	
15	Tx	9.0	60.0	15.3	1.2	5	60	340	97.8	87.8	13.6	F	25	12.0	
16	Tx	6.5	64.8	14.6	0.9	5	50	432	102.0	68.0	12.3	M	24	16.8	
17	Tx	15.4	61.0	10.7	0.7	6	50	2,093	100.0	89.0	11.8	F	192	15.0	
18	Tx	9.3	67.0	9.2	0.9	6	50	502	86.0	83.0	15.9	M	52	17.0	Av. $=13.2 \pm 2.2$
19	Nod. Tx	8.1	60.0	12.5	1.4	3	55	659	79.0	100.0	9.6	M	34	21.0	
20	Nod. Tx	12.0	58.8	7.0	0.8	7	50	620	100.0	82.0	10.2	F	26	22.0	
21	Nod. Tx	9.4	64.6	10.0	0.9	6	50	585	100.0	80.0	11.4	F	31	22.2	

* Eu. Ca. = Euthyroid-thyroid carcinoma.
Eu. Card. = Euthyroid-cardiac.
Tx = Diffuse toxic goiter.
Nod. Tx = Nodular toxic goiter.

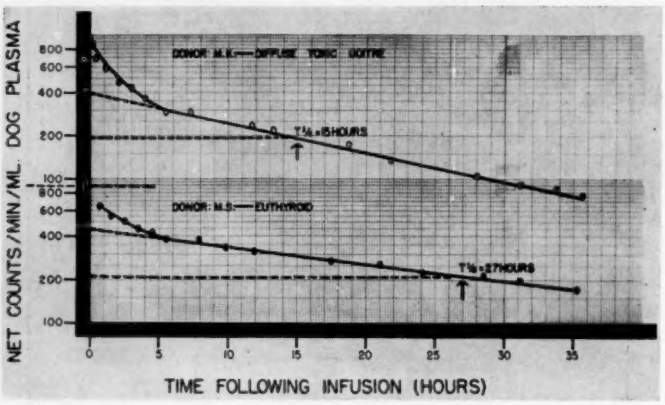

FIG. 1. COMPARISON OF THE EARLY DISAPPEARANCE IN DOGS OF ENDOGENOUSLY I-131-LABELLED THYROID HORMONE FROM A HYPERTHYROID VS. A EUTHYROID SUBJECT

These dosages delivered during the second 24 hours (12) 308 to 4,700 estimated R.E.P. β in the euthyroid group, and 670 to 1,365 R.E.P. β in the hyperthyroid patients. Following I-131 therapy, blood was removed into acid citrate after intervals (5 to 10 days in the euthyroids, 3 to 6 days in the hyperthyroids) shown by previous dialysis, trichloracetic acid precipitation, and butanol extraction studies to permit maximal incorporation of the I-131 into a plasma protein-bound and butanol-soluble (*i.e.*, thyroxin-like) form. Plasma was separated by centrifugation at 3,000 r.p.m. for 30 minutes. All procedures were carried out under sterile precautions. The butanol extractable fraction of the protein-precipitable I-131 was 76 to 96 per cent in the euthyroids and 68 to 100 per cent in the thyrotoxics.

Each plasma was then infused intravenously into an unanesthetized dog. At frequent intervals for the next 24 to 96 hours, blood was removed by femoral vein puncture from the recipient dog and the plasma radioactivity determined (13). Within 30 minutes following the infusion, the radioactivity of the recipient's plasma was virtually 100 per cent protein-precipitable.

The data were subjected to standard graphic analysis, as previously described (14), and as exemplified in Figure 1. Counts per minute per ml. of plasma were plotted as ordinate on semi-logarithmic paper against time following infusion as abscissa. Each resultant curve was resolved into straight line components—1) an initial steep slope for six to eight hours which could be resolved mathematically into several components followed by 2) a "slower" exponential component for the next 20 to 40 hours from which the half-times ($t\frac{1}{2}$) of the disappearance of the infused material from the circulation were determined.

B. *Results.* The results are presented in Table I. The half-times of disappearance in dogs of the radioactivity of the plasma obtained from euthyroid donors ranged from 21.0 to 37.6 hours, with an average of 29.5 ± 5.5 hours. In contrast, the half-times of disappearance of radioactivity of the thyrotoxic plasma ranged from 10.2 to 17.0 hours, with an average of 13.2 ± 2.2 hours. The difference is very highly significant statistically (the standard error (S.E.) of the difference between the means is 2.2, and "t" value is 7.41, and "p" is < 0.001).[5]

In preliminary observations, the half-times of disappearance in dogs of radioactivity of plasma from three patients with *nodular* toxic goiter fell within the euthyroid range—21.0, 22.0, and 22.2 hours. (See Table I.)

Series 2—Infusion of in vitro I-131-thyroxin-labelled human plasma—Disappearance rate in dog

A. *Methods.* Data identifying the donors and the plasma infused are presented in Table II. For each of seven paired experiments, plasma was obtained as described above from one euthy-

[5] Calculated for unpaired data with unequal variance and seven degrees of freedom.

roid subject and one patient with typical diffuse toxic goiter who had received no anti-thyroid therapy. An approximately equal amount of I-131-l-thyroxin [6] was added, under sterile precautions, to each plasma in a paired run. This varied from 1.0 to 20.0 μc., representing 0.22 to 14.9 μg., added to 12 to 22 ml. of plasma. After standing at room temperature for the same interval of time, which varied in different experiments, equal volumes of each plasma were infused into unanesthetized dogs. In Experiment No. 7, each dog received morphine sulfate (1.5 mg. per Kg.) 30 minutes before the infusion. The recipients' plasmas were analyzed at frequent intervals for residual radioactivity (see above). Following an initial rapid decrease in plasma radioactivity for six to eight hours, there was a slower exponential decrease for 30 to 48 hours from which the half-times of disappearance were obtained as described above and compared in each paired experiment.

B. *Results.* Data identifying the recipient dogs and the comparison of the disappearance rates of each paired experiment are presented in Table II. I-131-thyroxin, added to plasma, is almost immediately incorporated within the protein-precipitable fraction. In both the euthyroid and thyrotoxic plasmas infused, the radioactivity of aliquots was, within the limits of the methods, completely trichloracetic acid-precipitable and butanol-soluble. The euthyroid and hyperthyroid plasmas, therefore, could not be differentiated by these chemical procedures.

In contrast, in the first six experiments, the half-times of disappearance in dogs of the I-131-thyroxin-euthyroid plasmas were 17.0 to 30.2 hours, with an average of 23.5 ± 4.6 hours; those of the I-131-thyroxin-hyperthyroid plasmas were 10.4 to 15.0 hours, with an average of 12.7 ± 1.5 hours. The difference is highly significant—S.E. of the mean of the differences of the paired experiments equals 1.60, "t" equals 6.75, $p < 0.001$. In each paired experiment, the half-time of disappearance of the material obtained from the euthyroid donor was greater than that from the thyrotoxic patient; the ratios (euthyroid to hyperthyroid half-times) ranged from 1.3 to 2.4. In Experiment 7, in which each animal was pretreated with morphine sulfate, both disappearance

[6] The I-131-l-thyroxine and I-131-l-triiodothyronine were obtained from Abbott Laboratories. Each preparation contained approximately 1 to 6 per cent of the other labelled analogue.

TABLE II

Infusion of in vitro I-131-thyroxin-labelled human plasma into dogs

Expt. No.	Donor		Infusion				Recipient			Results	
	Thyroid status*	24-hr. I-131 uptake	Incubation period	Amount infused	I-131-thyroxin added		Sex	Wt.	Time followed	$t\frac{1}{2}$	Ratio of $t\frac{1}{2}$'s Eu/Tx
		%	(*hrs.*)	(*ml.*)	μc.	μg.		(*Kg.*)	(*hrs.*)	(*hrs.*)	
1	Eu	22.5	½	12	11.7	5.7	F	12.3	30	18.0	1.5
	Tx	71.0			11.7	5.7	F	12.3	45	12.0	
2	Eu	27.0	22	18	15.1	13.5	M	13.6	44	17.0	1.3
	Tx	75.0			16.7	14.9	M	15.9	44	13.0	
3	Eu	37.5	2	20	5.0	1.5	F	16.4	144	30.2	2.0
	Tx	73.8			5.0	1.5	M	16.8	144	15.0	
4	Eu	21.7	1½	20	1.0	0.41	M	11.8	48	24.5	2.1
	Tx	80.2			1.4	0.57	M	11.4	48	11.5	
5	Eu	17.0	3	20	3.5	0.22	M	15.0	96	26.5	1.9
	Tx	80.7			5.3	0.32	M	16.4	96	14.0	
6	Eu	18.0	¾	22	2.3	2.3	F	30.9	96	24.6	2.4
	Tx	76.7			3.1	3.1	M	29.5	96	10.4	
7	Eu	37.5	24	12	20.0	6.3	M†	13.6	76	32.5	1.5
	Tx	73.8			15.5	4.9	M†	12.7	76	22.0	

* Eu = Euthyroid.
Tx = Diffuse toxic goiter.
† Each dog received morphine sulphate 1.5 mg. per Kg. 30 minutes before the infusion.

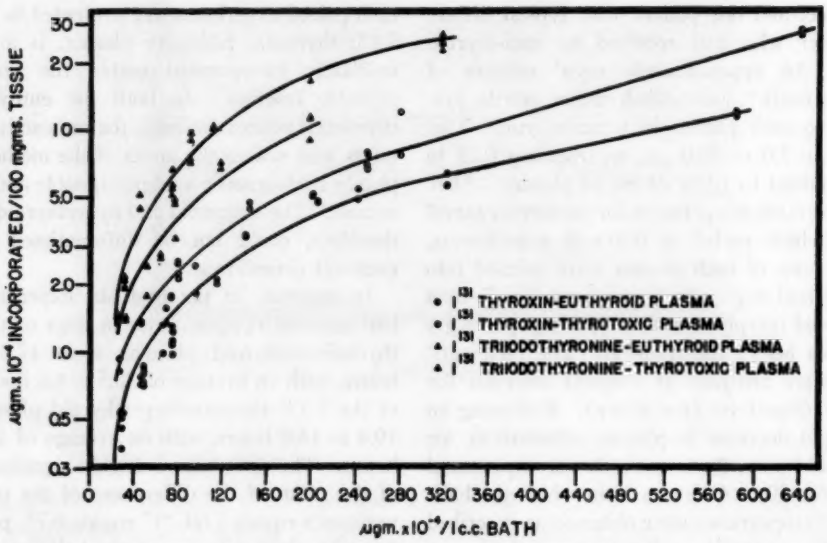

FIG. 2. FIVE HOUR *In Vitro* "UPTAKE" BY RAT DIAPHRAGM OF LABELLED THYROID HORMONE COMPONENTS FROM EUTHYROID AND HYPERTHYROID PLASMAS

Each symbol represents the average of two to four runs with a given plasma.

rates were slower than in untreated animals in the respective groups, but again the disappearance of the euthyroid-labelled plasma was distinctly slower than the hyperthyroid with a ratio similar to that of untreated animals.

Series 3—In vitro I-131-thyroxin- or I-131-triiodothyronine-labelled human plasma—Uptake by rat diaphragm

A. *Methods.* Varying amounts of I-131-l-thyroxin (I-131-l-THY) or I-131-l-triiodothyronine (I-131-l-TRI) were added to plasmas obtained as described above from euthyroid and untreated thyrotoxic patients. Three-ml. aliquots were then incubated with shaking at 37°C. under 95 per cent O_2–5 per cent CO_2 atmospheres in stoppered 10-ml. Erlenmeyer flasks for one-half to two hours. Female Sprague-Dawley rats, weighing 150 to 250 grams, fed on stock laboratory diet, were sacrificed by a sharp blow on the head and exsanguinated via a thoracic incision. The hemi-diaphragms were rapidly excised, weighed on a Roller-Smith torsion balance, and placed in a labelled plasma aliquot. When paired experiments were performed, one hemi-diaphragm was placed in the labelled euthyroid plasma, the other in the thyrotoxic plasma. The flasks were then shaken at 37°C. for varying periods, usually five hours, with replenishment of the 95 per cent O_2–5 per cent CO_2 atmosphere at one or two hourly intervals. Following this period, each hemi-diaphragm was removed, blotted, washed twice, each time for 20 seconds in 20 ml. 0.85 per cent NaCl solution, with interval and final blotting. The radioactive content was then determined by one of two methods: 1) a direct count of each hemi-diaphragm in a well-type scintillation counter, or 2) by dissolving the tissue in hot 2N NaOH, drying an aliquot, and counting with conventional end window G-M tubes (13). From the specific activity of the radioactive solutions, the "uptake" from the euthyroid and hyperthyroid plasmas was calculated as μg. incorporated per 100 mg. wet tissue. The percentage "uptake" per 100 mg. wet tissue was also determined by comparison with the radioactivity of the bath solutions before addition of the hemi-diaphragm. Each step was performed at least in duplicate.

To study the effect of the serum protein-bound iodine level, chromatographically pure sodium-l-thyroxin or l-triiodothyronine was added, in varying amounts, to euthyroid plasma, incubated at

TABLE III

In vitro rat diaphragm uptake of I-131-l-thyroxine and I-131-l-triiodothyronine from human plasma

Thyroid status	Diaphragm uptake (%/100 mg.) I-131-thyroxine	I-131-triiodothyronine
Euthyroid (20)	0.68 ± 0.11*	1.79 ± 0.15
Diffuse toxic goiter (15)	1.05 ± 0.08	2.64 ± 0.5
Nodular toxic goiter (8)	0.69 (range, 0.58–0.8)	1.8 (range, 1.3–2.4)

* Standard deviation.

room temperature (23 to 25°C.) or at 37°C. for 20 minutes to 24 hours, following which the rat diaphragm uptake of I-131-THY or I-131-TRI was determined, as above, and compared to control uptakes in unenriched plasma. Forty-seven such comparisons were made, in duplicate to quadruplicate, at enriched PBI levels of 9.0 to 20 μg. per cent.

B. *Results.* The absolute tissue radioactive content increased with increasing bath concentrations (Figure 2). The percentage "uptake," however, varied within a limited range over a wide range of bath concentrations. In 164 duplicate determinations, using euthyroid plasmas from 123 subjects, labelled by the addition of 15 to 585 $\times 10^{-4}$ μg. I-131-l-THY per ml., five hour "uptakes" ranged from 0.4 to 0.9 per cent per 100 mg. diaphragm, averaged 0.68 ± 0.13 per cent. In one determination, the uptake was 1.2 per cent. Similarly, in 140 runs, using euthyroid plasma from 109 subjects labelled by the addition of 12 to 475 $\times 10^{-4}$ μg. I-131-l-TRI per ml., "uptakes" ranged from 1.2 to 2.5 per cent per 100 mg. tissue, averaged 1.74 ± 0.27 per cent.

TABLE IV

Effect of addition of l-thyroxine (stable) to plasma on in vitro rat diaphragm uptake of I-131-l-thyroxine

Plasma PBI	Diaphragm uptake
μg./100 ml.	*%/100 mg.*
7.5 (control)	0.91; 0.91; 0.86
10.7	0.89; 0.91; 0.84
12.0	0.81; 0.76; 0.84
14.3	0.80; 0.85; 0.85

In 24 of 26 paired experiments with I-131-THY bath concentrations of 30 to 500 $\times 10^{-4}$ μg. per ml., the ratios of "uptakes" (hyperthyroid)/euthyroid, at the same bath concentration, ranged from 1.3 to 2.3 with an average of 1.7; in the remaining two runs, the ratio was 1.1. In 25 paired experiments with I-131-TRI (46 to 217 $\times 10^{-4}$ μg. per ml.), the ratios ranged from 1.3 to 2.3, with an average of 1.9. In one run, the ratio was 1.0. In preliminary experiments, the "uptakes" from eight *nodular* toxic goiter plasmas were in the euthyroid range (Table III).

The prior addition of chromatographically pure stable l-thyroxin or stable l-triiodothyronine to euthyroid plasmas to yield protein-bound iodine plasma concentrations comparable to those of the hyperthyroid state generally failed to increase significantly the subsequent percentage radioactive "uptake" by the rat diaphragm. A typical experiment is shown in Table IV. The various combinations of stable components added and subsequent radioactive "uptakes" are summarized in Table V. Thus, in 30 of 32 comparisons in which PBI levels were increased up to 18.4 μg. per cent by the addition of l-thyroxin, there was no significant increased per cent uptake. An increase to

TABLE V

The effect of prior enrichment of euthyroid plasma on diaphragm "uptake"

Stable component added	PBI levels attained	Radioactive label	No. of runs	Effect on % "uptake"/100 mg. tissue
	μg./100 ml.			
THY	8.6–17.4	I-131-THY	15	None
THY	10.4–18.4	I-131-TRI	15	None
	17.4		1	Increased from 2.0 to 3.1%
	15.8		1	Increased from 2.1 to 2.7%
TRI	8.0–15.0	I-131-THY	6	None
TRI	8.0–15.0	I-131-TRI	8	None
	15.2		1	Increased from 2.1 to 2.8%

hyperthyroid "uptake" levels of I-131 TRI was noted in two instances at PBI levels of 15.8 and 17.4 μg. per cent. In correlative studies, not included in Table V, increased uptakes were consistently obtained at PBI levels above 25 μg. per cent. The lowest level at which an increase was consistently effected was not determined. Quantitative data on triiodothyronine plasma levels in hyperthyroidism are not definitive. In 14 of 15 runs with PBI levels increased up to 15 μg. per cent by the addition of stable triiodothyronine, there was no significant increased per cent "uptake" of I-131-THY or I-131-TRI. In one experiment, an increase in PBI from 5.0 to 15.2 μg. per cent resulted in an increased "uptake" from 2.1 to 2.8 per cent.

DISCUSSION

The results of the three series of experiments show striking and consistent differences—1) thyroid hormone from patients with diffuse toxic goiter, labelled either endogenously by prior I-131 administration, or *in vitro* by the addition of I-131-tagged-thyroxin, and infused into dogs, disappeared more rapidly from the circulation than similarly labelled thyroid hormone from euthyroid subjects, and 2) greater amounts of radioactivity were incorporated *in vitro* by rat diaphragm from thyrotoxic plasma labelled *in vitro* with I-131-thyroxin or I-131-triiodothyronine than from similarly tagged euthyroid plasma, or from euthyroid plasma artificially enriched to hyperthyroid PBI levels.

These results are not adequately explained by prevailing concepts and indicate 1) a difference in the qualitative nature of the thyroid hormone-protein complex in hyperthyroidism as compared to the euthyroid state, or 2) the presence of a factor(s) in the plasma of the hyperthyroid individual differing qualitatively or quantitatively in its effect(s) on the disappearance from the circulation, or on tissue uptake, of the labelled hormone moiety.

Series 1 was based on the generally accepted concept that administered I-131 is taken up by the thyroid gland, incorporated therein into the thyroid hormone, which is released to circulate as a protein-bound hormone complex. It has been shown (8, 9, 15, 16) that the administration of very large doses of I-131 results in the release into the circulation of an abnormal I-131-labelled product, probably thyroglobulin, which is protein-precipitable but not butanol-soluble. Since it was therapeutically necessary to administer larger I-131 doses to our euthyroid subjects than to the patients with hyperthyroidism, the possibility had to be considered that the slower disappearance rate of the euthyroid plasma radioactivity in our studies was due to its content of an abnormally large, I-131-tagged thyroglobulin-like compound. However, the I-131 dosages used and the R.E.P. delivered to the donors' glands were, with the exception of Case 1, Series 1, below the range, that is, 3,600 R.E.P. β or more during the second 24 hours, found by Robbins, Rall, Becker, and Rawson (16) to be associated with the release of significant amounts of a thyroglobulin-like material into the circulation. Tubiana (17) found the butanol solubility of serum radioactivity of euthyroid patients receiving 50 to 100 mc. to be comparable to that following tracer doses.

The butanol solubility of the plasma was determined in six of the eight euthyroid subjects of Series 1. There was complete overlapping of these values with those of the thyrotoxic plasmas. Therefore, no significant amounts of a thyroglobulin-like fraction appear to have been released from the thyroid glands by the large dosages employed in our euthyroid group as compared to the thyrotoxic group. That the release of such a substance is probably not an important factor in the differences observed is further indicated by the consistent comparative results in Series 2 and 3 which are independent of I-131 radiation effect upon the donor's thyroid gland.

The possibility has been considered that the increased rate of disappearance or tissue "uptake" of the labelled thyrotoxic plasma might be due, in part, to its greater content of an unlabelled protein-bound iodine moiety, *i.e.*, a greater "saturation" of the specific binding plasma protein(s) by thyroxin or triiodothyronine with a resultant "spill-over" of the labelled component onto another plasma protein (*e.g.*, albumin) with a less firm binding capacity. Gross and Leblond (18) have shown, in the rat, that with greatly increasing dosage of infused I-131-thyroxin, from 10 to 2,000 times the estimated physiological range, there was a decrease in the percentage of the dose remaining in the plasma at given time intervals with a cor-

responding increase in the proportion found in the liver. Our results in Series 3 similarly indicate a positive correlation between the plasma concentrations of the labelled hormone component and the resultant absolute *in vitro* tissue uptake thereof.

Several recent studies have dealt with the problem of the binding capacity for thyroxin of serum, more specifically its thyroxin-binding protein ("TBP") or globulin ("TBG"). Albright, Larson, and Deiss (19) found this binding capacity to vary inversely with thyroid function, the hypothyroid binding more and the hyperthyroid less thyroxin than normal. The relationship between thyroxin added and that bound by "TBG" was roughly linear at concentrations of 0.05 to 0.4 μg. per ml., then reached a plateau. "Saturation" of "TBG" was reached at concentrations of approximately 0.15 to 0.2 μg. per ml. in the hyperthyroid as compared to 0.25 μg. per ml. in the euthyroid subject. There was distinct overlapping of the amounts bound by the euthyroid and hyperthyroid "TBG" at concentrations up to 0.2 μg. per ml. Robbins and Rall (20), in contrast, found that the concentration of thyroxin in α-globulin did not reach a plateau but continued to increase with increasing addition of thyroxin. The per cent thyroxin bound to alpha globulin at serum thyroxin concentrations of 0.059 to 0.12 μg. per ml. varied in an apparently random fashion between limits of about 60 and 80 per cent. After a critical evaluation of the significant limitations of the methods, they estimated that saturation of "TBP" occurs at serum thyroxin levels at least two to three times normal. They were not certain whether the values for thyroxin binding differed significantly in a single hyperthyroid serum from those obtained with normals. In a subsequent study using a method of reverse-flow zone electrophoresis, Robbins (21) found the thyroxin-binding capacity of the binding sites for thyroxin in the α-globulin in seven normal subjects to range from 0.16 to 0.25 μg. thyroxin per ml. serum, with a mean of 0.19. Freinkel, Dowling, and Ingbar (22) found that in all sera from hyper-, hypo-, and euthyroid subjects a small percentage of radioactivity migrated with albumin even at the lowest concentrations of thyroxin, and this progressively increased as the concentration of added thyroxin was augmented. They concluded, however, that because "TBP" had not been isolated, could not be measured directly, and had not been characterized as one or several proteins or binding-sites, more quantitative analysis was not justified. In further studies (23), these authors found that the *in vitro* "uptake" of radiothyroxin by slices of sheep, lambs, calves or beef heart, liver and kidney was dependent upon 1) the amount of tissue, 2) the amount of binding protein in the media, and 3) the concentration of thyroxin (stable plus labelled) in the media. Crispell, Kahana, and Hyer (24) found that the *in vitro* "uptake" of I-131-THY by red blood cells was increased by the addition of very large amounts of stable thyroxin (50 to 150 μg.) or triiodothyronine (100 μg.) to 2.0 ml. unwashed red blood cells. We have found (25) that prior addition of stable THY or TRI to whole blood increases the *in vitro* "uptake" of I-131-THY or I-131-TRI by the erythrocytes. This effect, however, increases with increasing time of prior incubation of the blood with the stable component and may not, therefore, be due solely to saturation of the binding protein(s), which effect is not time dependent.

Thus, precise quantitative relationships cannot be made at this time. Although it appears that available binding sites can be saturated at sufficiently high PBI levels, failure to obtain increased per cent *in vitro* "uptakes" by the enrichment of plasmas to PBI levels found clinically in hyperthyroidism lead us to conclude that our results are not adequately explained on this basis.

Furthermore, between our two groups in Series 1, there was distinct overlapping of the calculated specific activities of the infused material because of the varying concentrations of plasma radioactivity and amounts of plasma infused (Table I). It was estimated that the amounts of protein-bound iodine complex infused ranged from 1.8 to 3.6 μg. in the euthyroid group as compared to 3.2 to 9.0 μg. in the thyrotoxic group. There was no apparent correlation within either group between the estimated amount of the stable plus labelled protein-bound iodine complex in the infusion and the observed disappearance rate in the recipient.

Further evidence was obtained in Series 2 and 3 that the observed difference in disappearance rates between euthyroid and thyrotoxic plasma in Series 1 was not due solely to differences in the respective amounts of protein-bound iodine compounds. Despite the wide ranges of μg. and μc. of

added radioactive materials and the resultant wide ranges of specific activities of the infusions and baths in Series 2 and 3, the disappearance rates of radioactivity and the tissue "uptake" of radioactivity were consistently greater with thyrotoxic than with euthyroid plasmas under comparable experimental conditions. Total "uptake" of thyroxin (labelled plus unlabelled) from thyrotoxic plasma was more than twice that from labelled euthyroid plasmas containing comparable total amounts of thyroxin, calculated with the assumptions that the average PBI of euthyroids is 6.0 μg. per cent, that of the hyperthyroids is 15.0 μg. per cent and that these values represent chiefly thyroxin. Similar estimates of total triiodothyronine "uptake" cannot be made at present because of lack of data quantitating plasma triiodothyronine levels.

The results of the studies in nodular toxic goiter, although preliminary, are of interest. Although these patients could not be differentiated clinically or by standard laboratory methods from patients with diffuse toxic goiter, the disappearance rate of endogenously I-131-labelled thyroid hormone (three patients) and the tissue "uptake" of exogenously labelled plasma (eight patients) fell within the euthyroid range. Such results are consistent with the hypothesis that there is an overproduction of the normal thyroid hormone in certain hyperthyroid patients with a nodular goiter.

Available data are not sufficient to permit precise definition, in qualitative or quantitative terms, of the body compartment(s) involved in our infusion experiments. Extension of these studies to investigate the actual turnover of the infused component requires the establishment of conditions of equilibrium. In certain experiments in Series 1 and 2, the recipient's plasma radioactivity, beyond 48 hours, decreased at a third, slower exponential rate. Since the thyroid gland was not blocked in our experiments, it is possible that this is due, in part, to the addition to the plasma of "new" hormone, endogenously labelled in the dog's thyroid by the I-131 liberated in the catabolism of the infused, labelled hormone. Because this added hormone is not distinguishable by methods of this study from the infused test hormone, we have limited this analysis of infusion studies to the first 48 hours. Our results, therefore, are not to be interpreted in terms of the ultimate metabolism of the infused hormone, as has been done by Levy, Kelly, Cooper, and Jefferies (26) who demonstrated, in prolonged infusion studies, that the ultimate turnover was dependent upon the metabolic state of the recipient rather than that of the donor.

Despite this limitation, the differences observed between euthyroid and hyperthyroid plasmas under these varied experimental conditions appear significant. They raise the question of the validity of the concept that the essential abnormality in hyperthyroidism is the overproduction of the normal thyroid hormone. While it had been postulated that the circulating thyroid hormone was wholly a thyroxin-protein compound, the studies of Gross and Pitt-Rivers (2, 27, 28) demonstrating the natural formation and circulation of the calorigenically potent triiodothyronine, have necessitated a re-evaluation of this concept. We have found in preliminary studies (29) that the disappearance rate of I-131-l-triiodothyronine added to euthyroid plasma and infused into a dog is significantly greater than that of equivalent amounts of I-131-l-thyroxin added to an equal aliquot of the same plasma. Rawson *et al.* (30) have demonstrated, in euthyroid athyreotic humans, that I-131-triiodothyronine disappears from the plasma following intravenous administration at rates 2.4 to 4.8 times as rapidly as I-131-thyroxin. Sterling, Lashof, and Man (31) have obtained comparable results in euthyroid subjects. In Series 3, the rat diaphragm "uptake" of I-131-triiodothyronine was always greater than that of I-131-thyroxin at comparable bath concentrations of both euthyroid and hyperthyroid plasmas. Ratios of "uptake" (I-131-triiodothyronine to I-131-thyroxin) of 1.6 to 4.1, with an average of 3.0, were obtained in 31 runs in baths containing 30 to 340×10^{-4} μg. radioactive material per ml. We have also found (32) that, over a wide range of concentrations and incubation times, the "uptake" by rat diaphragm of I-131-l-triiodothyronine in buffer solutions is consistently greater (2.0 to 4.5 times) than that of I-131-l-thyroxin in comparable buffer solutions. Such findings suggest the possibility in hyperthyroidism of delivery to the tissues of a hormone complex containing relatively greater amounts of triiodothyronine or some other as yet unidentified potent component which enters peripheral tissues more rapidly and in greater amounts than does

thyroxin. This would not explain, however, our observations of greater disappearance rates and tissue incorporation of I-131-thyroxin from thyrotoxic as compared to euthyroid plasma.

Other possibilities which may be of pathogenetic importance in hyperthyroidism are 1) an abnormal linkage, in kind or degree, of the thyroxin or triiodothyronine to a plasma protein component(s), and 2) the effect of a plasma factor(s), either abnormal or present in increased amounts, which increases the rate of utilization of the hormone-protein complex, leading to a compensatory increase in production of the hormone by the thyroid gland to meet the increased demand. Such an effect might be specifically on the utilization of the thyroid hormone, or indirectly mediated. Relevant data on the effects of the infusion of heterologous plasma in animals are scanty. Sanford and Blackford (33) have reported that the injection, in dogs, of thyrotoxic sera or extracts of exophthalmic goiters produced a marked transient drop in blood pressure. Schoenborn (34), however, failed to find any difference in blood pressure response in cats following infusion of extracts of normal and thyrotoxic glands. Preliminary attempts to measure the blood pressure response in our unanesthetized dogs have been unsuccessful. Further studies are necessary to clarify these possibilities.

Experience with the methods used in this study has not been sufficient to permit evaluation of their possible use for diagnostic purposes. Extensive experience with tests of each single parameter of thyroid function has yielded overlapping results when applied to the broad spectrum of various levels of thyroid activity. The methods used in this study, however, do permit investigation of important parameters of thyroid function, concerning which pertinent data are meager. It is believed that their extension and integration with other methods in the over-all study of thyroid physiology and pathology are clearly indicated.

SUMMARY

1. Three series of experiments were performed to test the generally accepted concept of the biologically qualitative identity of the circulating thyroid hormone in the normal and hyperthyroid states.

2. The half-times of disappearance of endogenously I-131-labelled euthyroid human thyroid hormone during the first 24 to 48 hours following infusion into the dog ranged from 21.0 to 37.6 hours with an average of 29.5 ± 5.5 hours. In comparable experiments using labelled hormone from patients with diffuse toxic goiter, the half-times of disappearance ranged from 10.2 to 17.0 hours, with an average of 13.2 ± 2.2 hours. The difference was statistically very highly significant.

3. The half-times of disappearance in the dog of *in vitro*-labelled I-131-thyroxin euthyroid plasma ranged from 17.0 to 30.2 hours, with an average of 23.5 ± 4.6 hours; those for similarly labelled thyrotoxic plasma ranged from 10.4 to 15.0 hours with an average of 12.7 ± 1.5 hours. The difference was statistically highly significant.

4. Radioactivity of *in vitro*-labelled I-131-thyroxin-thyrotoxic plasma and I-131-triiodothyronine-thyrotoxic plasma was incorporated by the rat diaphragm *in vitro* in amounts averaging 1.7 and 1.9 times that of I-131-l-thyroxin-euthyroid plasma and I-131-triiodothyronine-euthyroid plasma, respectively.

5. The prior enrichment of euthyroid plasma by the addition of l-thyroxine or l-triiodothyronine to hyperthyroid PBI levels failed to increase the subsequent *in vitro* percentage radioactive "uptake" by the rat diaphragm to hyperthyroid levels.

6. In preliminary studies in patients with *nodular* toxic goiter, the half-times of disappearance of endogenously I-131-labelled thyroid hormone and *in vitro* rat diaphragm "uptakes" of I-131-l-thyroxin and I-131-l-triiodothyronine fell within the euthyroid range.

7. The results are not adequately explained by the prevailing concept that thyrotoxicosis with diffuse toxic goiter is caused solely by an excess of the normal thyroid hormone. They indicate 1) a qualitative difference in the nature of the circulating hormone-protein complex in hyperthyroidism as compared to the euthyroid state, or 2) a differential effect of a plasma factor(s) on the "peripheral utilization" of thyroid hormone in the two thyroid states.

8. Certain other theoretical implications of these concepts are discussed.

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THE EFFECTS OF NUCLEOSIDES ON THE RESISTANCE OF NORMAL HUMAN ERYTHROCYTES TO OSMOTIC LYSIS[1, 2]

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The investigations reported in this paper proceeded on the hypothesis that maintenance of the structural integrity of the human erythrocyte is dependent on continued production and utilization of energy by the cell. In an attempt to test this hypothesis the susceptibility of fresh human erythrocytes to osmotic lysis was studied in terms of the influence of various compounds that might serve as substrates for energy yielding reactions within the erythrocyte. Particular attention was paid to glucose (3) and purine nucleosides (4, 7) which have been shown to prolong the viability of stored erythrocytes and to retard their progressive lysis and diminished resistance to hypotonic solutions. It may be noted, however, that the effectiveness of the purine nucleosides in the preservation of erythrocytes has recently been questioned (8).

MATERIALS AND METHODS

Blood freshly drawn from normal adults was defibrinated and the erythrocytes were washed three times with about three volumes of isotonic sodium phosphate buffer (pH 7.3 to 7.4). Each washing was carried out with centrifugation in a Servall Refrigerated Angle Centrifuge for 10 minutes at 1,600 G and at 4° C. (SS-1 rotor). The phosphate buffer was prepared to contain 3.76 grams $NaH_2PO_4 \cdot H_2O$ and 11.62 grams Na_2HPO_4 in distilled water in a final volume of one liter. A fifty per cent suspension of the washed erythrocytes in isotonic sodium phosphate buffer was prepared, and two milliliters of this suspension were added to four milliliters of isotonic phosphate buffer containing the compound under study. This procedure resulted in a final 16.7 per cent suspension of erythrocytes. After thorough mixing, the suspensions were incubated in a waterbath at 37° C. for two hours, unless otherwise noted. Following incubation, the supernatant solution was removed after centrifugation in an International Clinical Centrifuge (about 1,600 G) for five minutes and the erythrocytes were washed twice with isotonic phosphate buffer. The erythrocyte suspensions were then restored to the original volume of six milliliters with isotonic phosphate buffer. Aliquots (0.2 ml.) of each suspension were added to two milliliter portions of pH 7.4 phosphate buffer solutions of varying hypotonicity and to distilled water. After mixing, these suspensions were allowed to remain at room temperature for 40 to 50 minutes, and then were centrifuged for five minutes in an International Centrifuge, Model SBV, size 1, at 2,000 RPM (810 G). One milliliter of supernatant solution was removed, diluted with four milliliters of water, and the extent of lysis was calculated from the amount of hemoglobin liberated as measured by the optical density at 541 mμ determined in a Beckman DU spectrophotometer.

In some experiments isotonic sodium chloride, 0.875 per cent, replaced the isotonic phosphate buffer. The freshly obtained erythrocytes were washed three times with isotonic sodium chloride and the preparation of the suspensions of erythrocytes, the incubation, and the post-incubation washings were carried out in a manner identical with the one employed with isotonic phosphate buffer. The extent of hemolysis was determined 40 minutes after the addition of 0.2-ml. aliquots of each suspension of erythrocytes to two milliliters of sodium chloride solution of varying hypotonicity and to distilled water. In the experiments in which the effect of inorganic phosphate was studied isotonic sodium phosphate buffer (pH 7.4) was mixed with isotonic sodium chloride to yield the desired concentration of phosphate.

Determinations of pH were made with a Beckman Model G glass electrode pH meter.

The concentrations of nucleosides were determined spectrophotometrically (9).

In several experiments smears of the erythrocyte suspensions were prepared at different stages and stained with Wright's stain. No morphological differences be-

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[3] Fellow of the National Foundation for Infantile Paralysis.

[4] Fellow of the American Cancer Society on the recommendation of the Committee on Growth of the National Research Council.

tween treated and untreated cells were observed, nor were there significant variations in hematocrit values.

Most of the purines, nucleosides and related compounds were obtained commercially. The purity of the purines and purine-containing compounds was determined by ultra-violet spectrophotometry and paper chromatography in suitable solvent systems. The 2,6-diaminopurine riboside (2,6-diamino-9-β-D-ribofuranosyl purine) and the adenine glucoside (9-β-D-glucopyranosyl adenine) were prepared by the method previously described (10). Samples of purine riboside (9-β-D-ribofuranosyl purine), benzimidazole riboside (1-β-D-ribofuranosyl benzimidazole), and 6-acetamido-9-tetraacetyl-β-D-glucopyranosyl purine were generously provided by Dr. George B. Brown. The Phenergan® (N-(2′-dimethylamino-2′-methyl)-ethylphenothiazine hydrochloride) was kindly supplied by Mr. Ambrose Hunsberger, Jr., of Wyeth Laboratories.

The special conditions of washing, incubation and osmotic lysis were chosen for the following reasons. No further increases in hemolysis were found to occur after exposure to hypotonic phosphate buffer solutions for 40 to 50 minutes at room temperature (20 to 25° C.). Since no differences in extent of lysis were noted in 15.0 per cent, 16.7 per cent, or 18.4 per cent suspensions of erythrocytes, the 16.7 per cent suspension was chosen. To control the effects of variation in the pH of the erythrocyte suspensions on the extent of hemolysis in hypotonic solutions (11), the cells were washed in isotonic phosphate buffer, pH 7.3 to 7.4, after incubation. Despite variations in pH from 7.0 to 8.2 during incubation with isotonic phosphate buffers of different pH, the effect on osmotic lysis of the pH alone could be eliminated by this post-incubation washing with pH 7.3 to 7.4 isotonic phosphate buffer (Table I). All experiments, unless otherwise noted, were performed with this control of pH, and with the length of exposure to hypotonic solution, and with the concentration of erythrocytes maintained constant.

RESULTS AND DISCUSSION

Of the various types of compounds studied, certain purine ribosides, on incubation with fresh normal human erythrocytes, were found to produce marked enhancement of resistance to osmotic lysis. The effective compounds were adenosine, guanosine, inosine, 2,6-diaminopurine riboside (2,6-diamino-9-β-D-ribofuranosyl purine) and xanthosine. Although incubation with adenosine or 2,6-diaminopurine riboside resulted in elevation of the pH of the suspension, washing the erythrocytes with isotonic sodium phosphate buffer (pH 7.3 to 7.4) brought all the suspensions to the same pH as the control suspension prior to their being added to the hypotonic phosphate buffer solutions. The results of a typical experiment are presented graphically in Figure 1. Xanthosine, inosine and adenosine increased the resistance of erythrocytes to osmotic lysis, whereas deoxyinosine had no effect. A minimal degree of protection resulted from incubation with ribose-5-phosphate and glucose. The deoxyribosides, ribose-5-phosphate, and glucose are discussed below.

The protective effect was maximal at concentrations of 6 and 10 micromoles of adenosine per milliliter of incubation suspension (Table II) and at about 8 micromoles of inosine per milliliter of incubation suspension (Table III). Because of the limited solubility of the other nucleosides, studies of their optimal concentration were not performed. Neither adenosine nor inosine had a significant effect on susceptibility to osmotic lysis when the two hour incubation was conducted at 4° C. The

TABLE I

Effect of pH on extent of hemolysis in hypotonic phosphate buffer after incubation for two hours in isotonic phosphate buffer *

pH of incubation buffer	7.0	7.0	7.4	7.4	7.8	7.8	8.2	8.2
pH of suspension after incubation	7.2	7.2	7.5	7.5	7.9	7.9	8.2	8.1
pH of washing buffer	7.0	7.4	7.4	7.4	7.8	7.4	8.2	7.4
pH of suspension after washing	7.1	7.4	7.4	7.4	7.8	7.4	8.2	7.4
Per cent of isotonicity	Per cent hemolysis							
46	17	5	4	4	2	5	2	6
43	57	21	22	21	6	20	5	22
40	85	63	62	62	22	60	16	60
37	95	88	87	88	63	87	44	86
34	98	94	94	96	87	95	73	95

* After incubation, half of the suspensions of erythrocytes was washed twice and re-suspended in isotonic phosphate buffer of the same pH as that of the buffer in which they were incubated, whereas the other half was washed twice and re-suspended in isotonic phosphate buffer, pH 7.4.

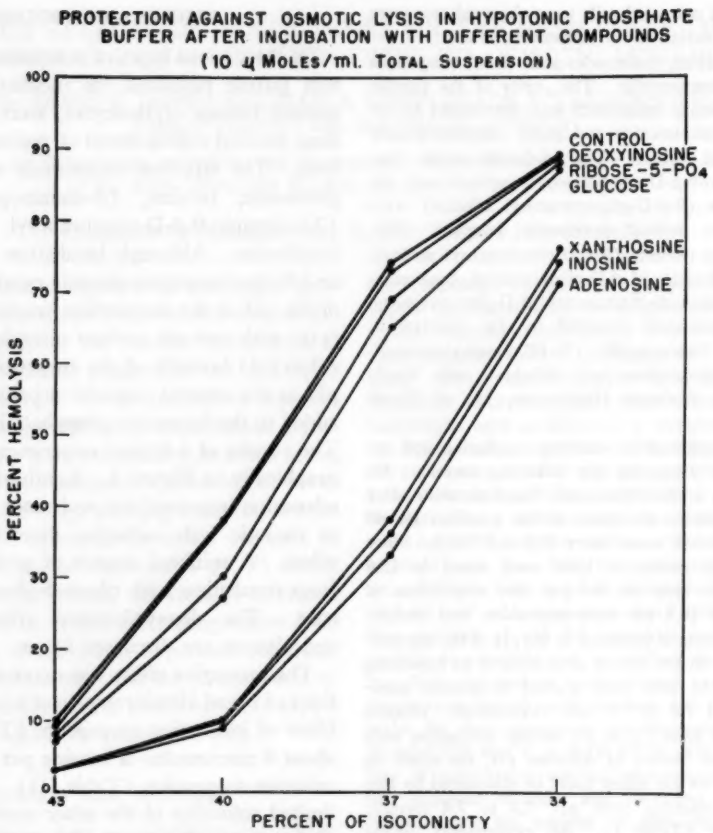

FIG. 1. FRESH HUMAN ERYTHROCYTES INCUBATED FOR TWO HOURS AT 37° C. IN ISOTONIC SODIUM PHOSPHATE BUFFER ALONE OR WITH 10 MICROMOLES PER MILLILITER OF VARIOUS COMPOUNDS AND SUBJECTED TO OSMOTIC LYSIS IN PHOSPHATE BUFFER SOLUTIONS OF VARYING DEGREES OF HYPOTONICITY

effect at 37° C. was considerably greater than at room temperature (Table IV).

The protective effect of adenosine or inosine was apparent after one hour of incubation with these compounds at 37° C. With longer periods of incubation, increased protection was observed. The effect of adenosine appeared less rapidly than that of inosine, but was somewhat more marked

TABLE II

Protection against osmotic lysis in hypotonic phosphate buffer after incubation for two hours with different concentrations of adenosine

Per cent of isotonicity	Control	Concentration of adenosine, μM/ml. suspension						
		1.1	1.4	2.1	4.1	6.1	10.0	20.0
		Per cent hemolysis						
100	2	3	2	1	2	1	2	2
40	10	12	8	6	4	4	4	4
37	30	28	24	19	12	10	10	9
34	49	49	45	41	30	27	26	30
31	67	67	66	62	57	52	51	54

TABLE III

Protection against osmotic lysis in hypotonic phosphate buffer after incubation for two hours with different concentrations of inosine

Per cent of isotonicity	Control	Concentration of inosine, μM/ml. suspension						
		1.0	1.9	4.1	5.8	7.8	9.9	21.5
		Per cent hemolysis						
100	1	1	1	2	2	1	2	2
43	9	4	2	2	1	2	3	3
40	29	21	14	6	5	5	6	9
37	68	62	47	29	24	20	25	30
34	88	87	82	72	67	68	69	71

TABLE IV

Effect of incubation temperature on the protection against osmotic lysis in hypotonic phosphate buffer after incubation for two hours with adenosine or inosine *

Per cent of isotonicity	Per cent hemolysis at incubation temperature								
	4° C.			24° C.			37° C.		
	Control	Adenosine	Inosine	Control	Adenosine	Inosine	Control	Adenosine	Inosine
46	5	5	4	7	3	4	8	3	2
44	15	13	12	17	10	10	21	4	5
42	35	31	30	39	22	23	44	10	13
40	62	58	56	67	49	48	71	25	31
38	82	80	78	86	74	73	86	49	59

* The concentration of adenosine was 10.0 and of inosine 9.9 micromoles per milliliter of incubation suspension.

after longer periods of incubation (two to four hours) (Figure 2).

In all studies in which osmotic resistance was enhanced there were considerable uptake and metabolism of the ribose moiety of the purine ribosides. Of the ribose metabolized, a major portion could be accounted for by increased lactate production. The details of these investigations are reported elsewhere (12).

When isotonic sodium chloride solution was substituted for the isotonic sodium phosphate buffer, incubation with adenosine, deoxyadenosine, guanosine, or 2,6-diaminopurine riboside at concentrations of two micromoles per milliliter increased resistance to osmotic lysis in hypotonic sodium chloride, whereas inosine and xanthosine were ineffective (Table V). However, except for guanosine, the compounds that exerted a protective effect in the sodium chloride system raised the post-incubation pH to 7.8, an alteration that could not be reversed completely by washing the erythrocytes with isotonic sodium chloride. When adenosine, deoxyadenosine, or 2,6-diaminopurine riboside was employed at a concentration of 10 micromoles per milliliter, the elevation of the pH was even more striking and attained values of 8.1 to 8.5. The pH of the control incubated in isotonic sodium chloride alone was 7.5 to 7.6 after incubation. Since elevation of the pH is known to inhibit hemolysis (11), the protective effects of

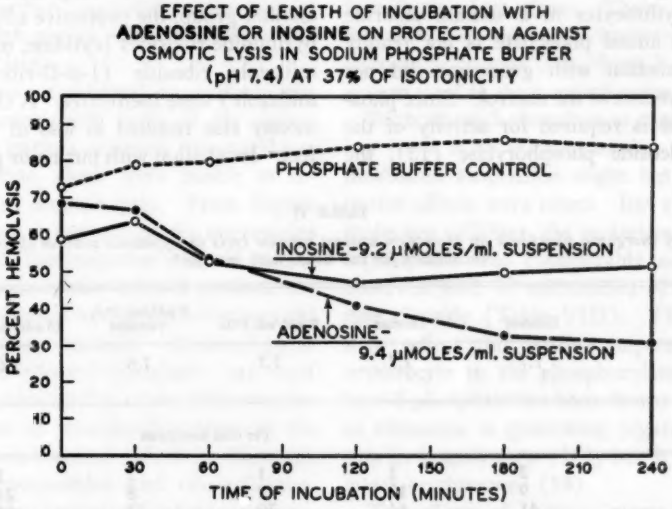

FIG. 2. EXTENT OF HEMOLYSIS OF FRESH HUMAN ERYTHROCYTES IN PHOSPHATE BUFFER AT 37 PER CENT OF ISOTONICITY AFTER INCUBATION IN ISOTONIC SODIUM PHOSPHATE BUFFER ALONE OR WITH ADENOSINE OR INOSINE FOR DIFFERENT LENGTHS OF TIME AT 37° C.

TABLE V

Protection against osmotic lysis in hypotonic sodium chloride after incubation for two hours with purine nucleosides *

	Control	Adenosine	Deoxy-adenosine	Guanosine	Inosine	2,6-DAPR†	Xanthosine
pH of suspension after incubation	7.6	7.8	7.8	7.6	7.6	7.8	7.3
pH of suspension after washing	7.5	7.6	7.6	7.4	7.4	7.7	7.3
Per cent of isotonicity	Per cent hemolysis						
51	4	2	2	5	3	3	9
49	12	4	4	7	11	8	25
47	31	6	9	21	33	9	43
45	57	16	26	39	55	31	59
43	80	37	49	67	78	36	80

* Concentration of compounds was two micromoles per milliliter of incubation suspension.
† 2,6-diaminopurine riboside.

adenosine, deoxyadenosine and 2,6-diaminopurine riboside in the sodium chloride medium might be the result of the change in pH alone. Inosine, ineffective in the sodium chloride medium whose pH it did not alter, became effective on the addition of as little as 18 micromoles of inorganic phosphate per milliliter of incubation suspension (Table VI). These latter observations are compatible with the finding that erythrocyte nucleoside phosphorylase requires phosphate or arsenate for its action (13, 14).

The effect of guanosine in enhancing osmotic resistance of erythrocytes in a sodium chloride medium without added phosphate is not readily explained. Incubation with guanosine did not raise the pH above that of the control. Since phosphate or arsenate is required for activity of the erythrocyte nucleoside phosphorylase (13), the reason for the effectiveness of guanosine in this system without added phosphate is obscure.

In order to investigate the mechanism by which the purine nucleosides exert their effects on the susceptibility of human erythrocytes to osmotic stress, many related compounds were studied. When the nucleotides listed in Table VII (adenosine monophosphates, adenosine diphosphate, adenosine triphosphate, inosine-5′-phosphate) were employed, there was no enhanced protection against osmotic lysis. When the purine portion of the effective compounds was replaced by another group, the protective effect was lost. The pyrimidine ribosides (cytidine, uridine) and benzimidazole riboside (1-β-D-ribofuranosyl benzimidazole) were ineffective. A change in the sugar moiety also resulted in loss of the protective effect. Incubation with purine or pyrimidine deoxy-

TABLE VI

Effect of inorganic phosphate on protection against osmotic lysis in hypotonic sodium chloride after incubation for two hours with inosine *

	Control	Inosine	9 μM/ml. PO_4	9 μM/ml. PO_4 +inosine	18 μM/ml. PO_4	18 μM/ml. PO_4 +inosine
pH of suspension after washing	7.7	7.7	7.7	7.6	7.6	7.6
Per cent of isotonicity	Per cent hemolysis					
100	2	1	1	2	1	1
49	9	12	22	8	23	3
46	41	44	70	42	64	22
43	79	76	85	81	74	75
40	91	91	91	89	91	86

* Concentration of inosine was eight micromoles per milliliter of incubation suspension.

TABLE VII

Compounds ineffective in enhancing resistance to osmotic lysis in hypotonic pH 7.3 to 7.4 sodium phosphate buffer

Compound	Micromoles*
Nucleotides	
Adenosine-2′-phosphate	10
Adenosine-3′-phosphate	10
Adenosine-5′-phosphate	10
Adenosine diphosphate	10
Adenosine triphosphate	10
Inosinic acid	10
Pyrimidine ribosides	
Cytidine	10
Uridine	10
Purine deoxyribosides	
Deoxyadenosine	2, 10
Deoxyinosine	2, 10
Deoxyguanosine	2, 9.7
Pyrimidine deoxyribosides	
Thymidine	10
Deoxycytidine	10
Related compounds	
Benzimidazole riboside	6
Adenine glucoside	10
Purine riboside	10
Purines	
Adenine	2, 4
Hypoxanthine	2
2,6-diaminopurine	6
Intermediates of carbohydrate metabolism	
Sucrose	10, 25, 50
Fructose	10
Galactose	10
Glucono-lactone	10
Ribose	4, 10
Deoxyribose	10
Glycolic acid	10
Sodium pyruvate	10
Sodium lactate	10, 20
Glucose-1-phosphate	10
Glucose-6-phosphate	10
Fructose-1,6-diphosphate	10
3-phosphoglyceric acid	10
2,3-diphosphoglycerate	6
Tricarboxylic acid cycle compounds	
Oxaloacetic acid	10
Succinic acid	10
Fumaric acid	10
Malic acid	10
Citric acid	10
Miscellaneous compounds	
Glycine	10
Methionine	10
Cysteine	10
Glutamic acid	10
Aspartic acid	10
δ-aminolevulinic acid	10
Glutathione	10
Nicotinamide	10
Oxythiamine	1
Promethazine	0.16, 0.4
Sodium acetate	10, 20, 30
Sodium chloride	10

* Concentration in micromoles per milliliter of incubation suspension.

ribosides (deoxyadenosine, deoxyinosine, deoxyguanosine, thymidine, deoxycytidine) or adenine glucoside (9-β-D-glucopyranosyl adenine) failed to enhance resistance to osmotic lysis. The specificity of the phenomenon of protection against osmotic lysis was further supported by the lack of effect observed with purine riboside (9-β-D-ribofuranosyl purine) itself.

Experiments were conducted to evaluate the influence of the component parts of the purine nucleosides. The purines, adenine, hypoxanthine, or 2,6-diaminopurine, alone were unable to increase resistance to osmotic lysis. From Figure 3 it is apparent that a mixture of six micromoles per milliliter of 2,6-diaminopurine with six micromoles of ribose-5-phosphate did not produce the protective effect afforded by an equivalent amount of 2,6-diaminopurine riboside. Ribose-5-phosphate, rather than ribose-1-phosphate, was used because of the extreme lability of the latter and because the presence of phosphoribomutase in the human erythrocyte is inferred (15, 16). Mixtures of adenine or hypoxanthine and ribose-5-phosphate, even when the pentose phosphate was present in a concentration eleven to twenty-two times that of the purine, were also ineffective. Incubation of fresh human erythrocytes with a purine and ribose-5-phosphate resulted in no greater protective effect than the slight effect observed with ribose-5-phosphate alone. The effect of a purine riboside plus ribose-5-phosphate plus a purine was no greater than that of a purine riboside plus ribose-5-phosphate alone. These findings indicate that there was little or no formation of nucleosides from added purine plus ribose-5-phosphate in the system used in these experiments.

With ribose-5-phosphate at concentrations ranging from 9 to 90 micromoles per milliliter of incubation suspension slight but increasing protective effects were noted. But even at 90 micromoles per milliliter, the protective effect of ribose-5-phosphate was considerably smaller than that observed with 10 micromoles of an effective purine riboside (Table VIII). This limited effect may reflect the relative impermeability of the erythrocyte to the phosphorylated pentose. Ribose-5-phosphate has been shown to be as effective as adenosine in generating organic phosphate esters in hemolysates (17), but it is ineffective in intact erythrocytes (18).

The effects of various intermediates of carbohydrate metabolism were studied. Glucose provided a moderate degree of protection against osmotic lysis. This effect was independent of the

concentration in a range of 5 to 100 micromoles per milliliter, perhaps as a result of the limited ability of the erythrocyte to utilize this sugar *in vitro* (Table IX). A disaccharide (sucrose), other hexoses (fructose, galactose, glucono-lactone), pentoses (ribose, deoxyribose), glycolic acid, sodium pyruvate, and sodium lactate were unable to enhance resistance to osmotic stress in the concentrations employed (Table VII). The phosphorylated intermediates of carbohydrate metabolism listed in Table VII were also ineffective, probably as a result of impermeability of the erythrocyte to phosphorylated sugars. Although the mature mammalian erythrocyte does not possess an intact tricarboxylic acid cycle, several of the enzymes of this cycle are known to be present (19). However, compounds of the cycle (citric, fumaric, malic, oxaloacetic and succinic acids) were unable to increase osmotic resistance.

A number of metabolites or agents unrelated in structure to purine ribosides were also studied. Several amino acids (aspartic acid, cysteine, glutamic acid, glycine, methionine), nicotinamide, glutathione, delta-aminolevulinic acid, sodium acetate and sodium chloride did not enhance resistance to osmotic lysis. Phenergan®, reported by Schales to inhibit the progressive increase in osmotic fragility observed in whole blood stored with acid citrate-dextrose solution (20), was ineffective in the system used in these studies.

These experiments on freshly drawn human erythrocytes as well as the studies on stored

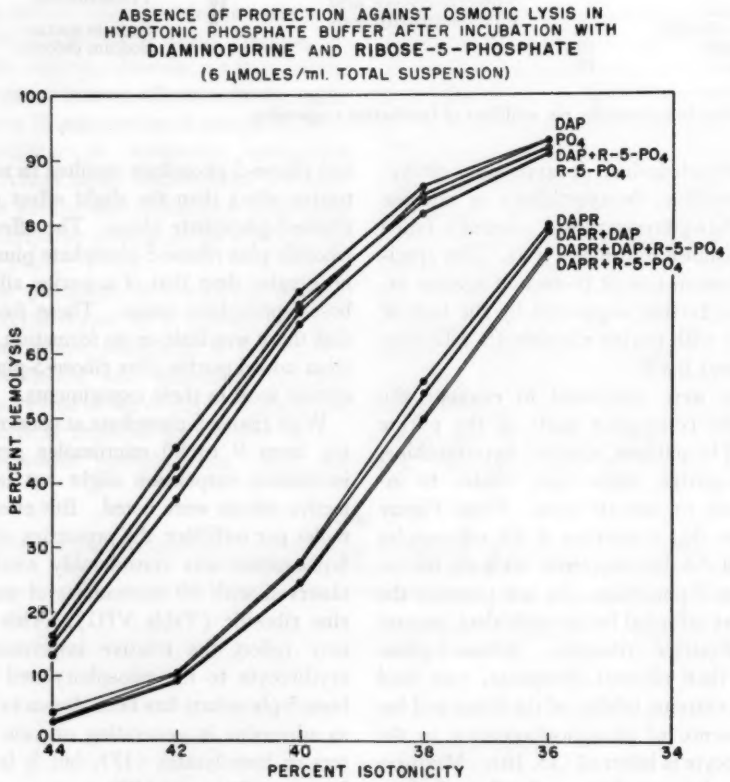

FIG. 3. EXTENT OF HEMOLYSIS OF FRESH HUMAN ERYTHROCYTES IN HYPOTONIC SODIUM PHOSPHATE BUFFER AFTER TWO HOURS INCUBATION AT 37° C. IN ISOTONIC PHOSPHATE BUFFER ALONE OR WITH 2,6-DIAMINOPURINE (DAP), RIBOSE-5-PHOSPHATE (R-5-PO_4), 2,6-DIAMINOPURINE RIBOSIDE (DAPR), OR VARIOUS COMBINATIONS OF PURINE, PURINE RIBOSIDE, AND PENTOSE PHOSPHATE

TABLE VIII

Protection against osmotic lysis in hypotonic phosphate buffer after incubation for two hours with different concentrations of ribose-5-phosphate

Per cent of isotonicity	Control	Inosine*	Concentration of ribose-5-PO_4, μM/ml. suspension: 4.5	9.0	22.5	45.0	67.5	90.0
			Per cent hemolysis					
44	11	4	11	9	8	7	6	6
42	34	8	32	28	27	21	20	18
40	61	21	60	55	54	47	44	42
38	80	46	84	80	78	74	73	70
36	90	74	92	92	92	90	90	88

* Concentration of inosine was 10 micromoles per milliliter of incubation suspension.

erythrocytes (4, 21–24) demonstrate that purine ribosides are an effective vehicle for introduction of ribose phosphate into the human erythrocyte. In correlative studies (12) it was shown that the ribose phosphate which is taken up by the erythrocyte can be accounted for to a considerable extent by an increased production of lactic acid. The reaction sequence for adenosine involves an initial deamination to inosine by an adenosine deaminase present in the erythrocytes of man and other species (23, 25). Phosphorolytic cleavage of the resulting inosine by nucleoside phosphorylase yields hypoxanthine and ribose-1-phosphate. Ribose-1-phosphate probably enters the hexose monophosphate shunt via conversion to ribose-5-phosphate by phosphoribomutase whose presence in human erythrocytes is strongly inferred (15, 16). The ribose-5-phosphate can then be metabolized via the hexose monophosphate shunt and the Embden-Meyerhof pathway to lactic acid.

The nucleoside phosphorylase of the erythrocyte appears to be specific for inosine or guanosine (13). The requirement of this enzyme for inorganic phosphate would explain the lack of increased resistance to osmotic lysis when erythrocytes were incubated with inosine in isotonic sodium chloride solution and the appearance of enhanced resistance when inorganic phosphate was added to the medium. The reactions involved in the utilization of xanthosine or 2,6-diaminopurine riboside are not yet known but probably entail amination and deamination, respectively.

The mechanism for transfer of ribose phosphate into the erythrocyte by means of purine ribosides has considerable specificity. One may speculate that an effective purine riboside must have a specific structure which enables it to permeate or to be bound to the cell membrane in such fashion that the deaminase or nucleoside phosphorylase can readily react with it. Once the ribose has been cleaved phosphorolytically from the purine, the purine, in large part and perhaps wholly, is released to the environment of the cell (23, 26).

The subsequent metabolism of the ribose phosphate to lactic acid requires that at least a major portion of the hexose-monophosphate shunt and the Embden-Meyerhof pathway be intact. To determine whether the mechanisms involved in the protective effects of the purine ribosides may decline with aging of the erythrocyte, as do some enzymatic activities (27), the relationship of the age of the erythrocyte *in vivo* and on *in vitro* storage to the effects of purine ribosides on osmotic resistance has been investigated (28). The results of these studies indicate that the enhancement of osmotic resistance by inosine is greater in young than in old erythrocytes of the rabbit which have aged *in vivo*. They show also that human erythrocytes, stored in acid-citrate-dextrose solution at 4° C., have an increased osmotic resistance after incubation with inosine. This effect, however, declines progressively with prolonged storage.

The effectiveness of the purine ribosides in enhancing osmotic resistance of fresh erythrocytes as well as in prolonging survival of stored erythrocytes suggests that this system for the study of osmotic lysis may provide a useful technique for screening agents which may be effective in the preservation of blood.

It is tempting to speculate on the mechanisms

TABLE IX

Protection against osmotic lysis in hypotonic phosphate buffer after incubation for two hours with different concentrations of glucose

Per cent of isotonicity	Control	Adenosine*	Concentration of glucose, μM/ml. suspension: 5	10	25	50	100
			Per cent hemolysis				
42	10	4	7	6	6	6	7
40	27	6	17	16	17	16	19
38	59	16	47	46	44	44	46
36	84	38	75	74	73	74	76
34	93	69	91	91	91	90	92

* Concentration of adenosine was 10 micromoles per milliliter of incubation suspension.

by which the purine ribosides enhance osmotic resistance. It is known that the purine ribosides can promote the production of organic phosphate esters (4, 21, 24), the retention of potassium (29), and the extrusion of sodium (30) in human erythrocytes. Enhanced osmotic resistance may reflect changes in the hydration of the erythrocyte which may result from these changes in organic phosphates and in electrolytes. Additional work is required, however, to determine whether the enhanced osmotic resistance may be associated with an active transport of water or with primary changes in the permeability of the erythrocyte membrane which are reflected in the movements of the electrolytes.

SUMMARY AND CONCLUSIONS

The resistance of fresh, normal human erythrocytes to osmotic lysis can be enhanced by incubation with the purine ribosides, adenosine, guanosine, inosine, 2,6-diaminopurine riboside, or xanthosine in an isotonic sodium phosphate buffer at pH 7.3 to 7.4. This effect was also observed, but to a much lesser extent, on incubation of erythrocytes with glucose or ribose-5-phosphate.

It is suggested that the metabolism of purine ribosides *in vitro* provides energy which may be utilized in maintenance of the structural integrity of the erythrocyte subjected to osmotic stress.

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OVERPRODUCTION OF URIC ACID AS THE CAUSE OF HYPERURICEMIA IN PRIMARY GOUT

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Glycine contributes carbon atoms 4 and 5 and nitrogen atom 7 of the purine ring (1–3). Benedict, Yü, Bien, Gutman and Stetten (4) have described experiments in which the abundance of N^{15} in uric acid was measured for several days following an oral test dose of glycine-N^{15}. In two gouty subjects, three times as much of the administered N^{15} was recovered in urinary uric acid as in normal subjects. In two others, however, normal quantities of N^{15} appeared in uric acid. In these studies, there was a rank order correlation between the daily excretion of uric acid in the urine and the per cent of ingested isotope appearing in urinary uric acid. In subjects who exhibited abnormally high basal uric acid excretions, an abnormally rapid and excessive incorporation of dietary glycine into uric acid occurred. In others whose basal excretion was approximately normal, utilization of glycine for uric acid synthesis was normal. Muller and Bauer (5) and Bishop, Rand, and Talbott (6) have each reported an additional gouty patient excreting normal quantities of uric acid who incorporated normal quantities of glycine-N^{15} into urinary uric acid. The latter authors also described one gouty patient who incorporated excessive quantities of glycine-N^{15} into urinary uric acid on two of three occasions despite a normal urinary urate excretion. These studies, therefore, demonstrated that overproduction of uric acid was present in some gouty subjects, but failed to give a clear indication of the cause of hyperuricemia in another, perhaps predominant group of the gouty population.

Recently, we have administered glycine-1-C^{14} to control and gouty patients in order to label urinary purines, as part of a study of intermediates in urate synthesis (7). The first two gouty patients both exhibited excessive incorporation of C^{14} into urinary urate, one despite a normal urate excretion (7, 8). It was therefore decided to re-investigate the problem of the rate of generation of uric acid in gout, employing tracer doses of glycine-1-C^{14} rather than large doses of glycine-N^{15} as were required in previous studies.

In the present study, glycine-1-C^{14} was administered orally in 2.5 to 25 μc. doses to control and gouty subjects, and the concentration and cumulative incorporation of isotope into urinary uric acid were determined. This paper presents data indicating that overincorporation of glycine-1-C^{14} into uric acid is a consistent finding in primary gout, and that the failure of the glycine-N^{15} technique to disclose overincorporation in some gouty subjects may have been the result of the large dose of glycine employed. These results have been interpreted as showing that the hyperuricemia of primary gout is due to overproduction of uric acid in the majority if not all gouty subjects.

METHODS

Glycine-1-C^{14}, specific activity 1 to 2 mc. per mM, was purchased from Nuclear Instrument and Chemical Company. Uric acid determinations were performed by differential spectrophotometry according to Praetorius (9), employing purified uricase purchased from Worthington Biochemical Corporation. Uric acid was isolated from urine either by adsorption onto charcoal and subsequent elution with alkali (10) or by precipitation with copper (11). It was recrystallized from lithium carbonate solution by addition of acetic acid (12). Uric acid was transferred to stainless steel planchets as an acetone suspension, dried under an infra-red lamp, and counted in a Robinson gas flow counter (13) having a background of four counts per minute. Self-absorption corrections were made by referring all counting values to a standard mass of 3.3 mg. per 1.54 $cm.^2$ planchet area under which condition absolute counting efficiency was 24 per cent. All specific activity values in this paper have been normalized to correspond to a standard dose of 5.0 μc. of glycine-1-C^{14}.

All subjects were maintained on a purine poor diet containing about 55 Gm. of protein for three to five days before administration of glycine and thereafter for the duration of the study. This diet was calculated to contain an average of 30 mg. of purine-N per day. When the urinary uric acid level had reached a constant minimal value, 2.5 to 25 μc. of glycine-1-C^{14} (0.2 to 2.0 mg.)

TABLE I

Data on subjects of study

Name Age, Race Wt. (Kg.)	Diagnosis	Hemogram Hct. %	Hgb. Gm. %	WBC per mm.³	Renal status BUN mg. %	NPN mg. %	Urinalysis	Uric acid Serum mg. %	Urine mg./day ± S.D.	Glycine-1-C^{14} μc.	Clinical résumé
C. L. 62, W. 67.9	Osteoarthritis, rt. hip (10 years)	45		9,500	19		Normal with S. G. 1.018	4.1–4.4	355 ± 42	5.0	
W. W. 21, W. 67.3	Duodenal ulcer (9 months)	42	15.2	4,850		34	Normal with S. G. 1.024	4.4	346 ± 50	13.6	No stigmata or family history of gout or of proliferative hematopoietic disorders.
H. B.* 26, C. 62.7	Contact dermatitis		14.0	8,700		27	Normal with S. G. 1.021	3.8	231 ± 66	25.0	
R. A. 32, C. 62.8	Duodenal ulcer, asymptomatic hyperuricemia	42	13.8	6,100		37	Normal with S. G. 1.028	7.6–7.8	343 ± 31	14.1	No family or personal history of articular disease or of renal disease.
O. N.† 61, W. 80.9	Non-tophaceous gout	50		8,600	20		Normal with S. G. 1.018	I 8.3–8.8 II 6.9–7.2	435 ± 42 391 ± 51	5.0 4.3	Thirteen-year history of recurrent gout, average of 2 attacks per year. Mild hepatomegaly. One-year interval between studies.
E. H.‡ 52, W. 89.4	Non-tophaceous gout	45	15.7	9,100	15		Normal with S. G. 1.014 P.S.P. 74% in 2½ hours	8.8 → 4.5	1,054 ± 196	5.0	Three acute attacks of gout, 5, 2, and ½ years prior to study. Probenecid therapy (see footnote).
D. W.§ 58, W. 93.1	Benign parotid tumor, tophaceous gout, chronic alcoholism	44	14.5	10,500 → 6,700	12		Normal with S. G. 1.022 P.S.P. 71% in 2½ hours	8.0–8.6	317 ± 25	2.5	Eleven-year history of recurrent gout, 5 attacks per year. BSP retention (27 and 19%). Developed tophus 1 month following study.
V. L.† 54, C. 100.0	Early tophaceous gout		14.2	9,300	14		Normal with S. G. 1.014	I 8.4 II 8.2	458 ± 48 404 ± 67	10.0 15.0	Seven-year history recurrent gout. Acute attack during first study. Developed tophus of finger shortly thereafter. BP 160/112. Four-month interval between studies.
W. R. 65, W. 71.8	Non-tophaceous gout, pyelonephritis	42	14.0	14,100 → 7,600		37	Max. urine conc. 1.015 P.S.P. 35% in 2 hours	6.3–6.5	313 ± 61	25.0	Eight-year history of recurrent gout. Admitted with acute pyelonephritis (Proteus vulgaris). Later, I.V.P. = poor excretion in 10 minutes. C^{14} study during convalescence.
H. H.* 62, C. 102.8	Non-tophaceous gout	38	12.2	8,850		37	Normal with S. G. 1.023 P.S.P. 55% in 2 hours	7.7–8.0	685 ± 82	25.0	Attacks of gout 1 year and again 1 month prior to study. Unexplained hyperglobulinemia of 6 Gm. %. BP 178/116.

* Only three and four days of urine were collected on H. B., and H. H., respectively, following C^{14}-glycine. These data are not included in Figures 1 and 2.

† In the second studies on O. N. and V. L., 0.1 Gm. of unlabeled glycine was given per Kg. of body weight, along with glycine-1-C^{14}.

‡ This patient was studied while on probenecid 1.0 Gm. per day. For some months prior to the study, his serum uric acid level was stable at 4.5 to 4.8 mg. per cent, and his average uric acid excretion during the glycine-1-C^{14} study was similar to pre-treatment excretion values (822 and 1,026 mg. per day). He was therefore judged to have mobilized and excreted the excess miscible uric acid and once again to be in a dynamic steady state with respect to uric acid synthesis and excretion.

§ Respiratory $C^{14}O_2$ excretion was measured on this patient, also.

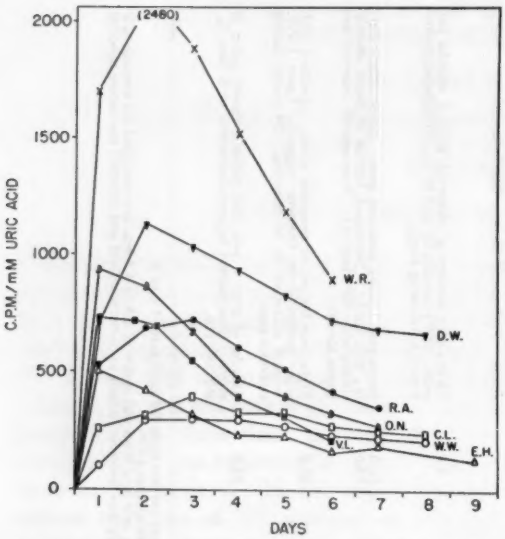

FIG. 1. THE CONCENTRATION OF C^{14} IN DAILY URINARY URIC ACID FOLLOWING ORAL ADMINISTRATION OF GLYCINE-1-C^{14}

The specific activity of uric acid has been plotted against time in days for two control (W. W. and C. L.), one asymptomatic hyperuricemic (R. A.), and five clinically gouty subjects. The specific activity values have been normalized to correspond to a standard dose of 5.0 μc. of glycine-1-C^{14}.

were administered orally in solution with a light breakfast. Daily urine collections were obtained under toluene at room temperature for varying periods after ingestion of labeled glycine. Pertinent data on the subjects of this study are given in Table I.

In one gouty subject (D. W.), the labeling of expiratory CO_2 was determined by methods previously described (14). Uric acid degradations were performed according to Buchanan, Sonne, and Delluva (2). C-6 and C-(2+8) were counted as $BaCO_3$. C-(4+5) were counted as glyoxylic acid semi-carbazone.

RESULTS

The concentrations of C^{14} in urinary uric acid following the ingestion of a single tracer dose of glycine-1-C^{14} are plotted as a function of time in Figure 1. In two control subjects, C. L. and W. W., the specific activities reached maxima on the third day, after which there was a gradual decline in isotope concentration. However, in a third control subject, H. B.,[1] maximal enrichment occurred in the sample excreted 6 to 17 hours following glycine-1-C^{14} administration and was then 510 c.p.m. per mM of uric acid. In the seven hyperuricemic subjects, comprising one patient with asymptomatic hyperuricemia and six with clinical gouty arthritis,[1] there was prompt isotopic enrichment of urinary uric acid, such that the first day specific activity values were up to three and one-half times greater than those found in the control subjects. However, because of differences in quantities of uric acid excreted, concentration data alone do not clearly separate the gouty and control subjects. In the asymptomatic hyperuricemic subject, the specific activity curve was not maximal until the third day but was approximately twice the level of enrichment of control curves throughout. Thus, the day of maximal enrichment also does not clearly separate a gouty from a control subject. In the six clinically gouty subjects, maximal specific activity values were encountered on either the first or second day, and there was a general similarity in the shapes of the enrichment curves of the gouty subjects. There was, however, a five-fold range in the heights of the maximal specific activity values which varied from 510 to 2480 c.p.m. per mM of uric acid.

From knowledge of the quantity of uric acid in each day's urine, and of its C^{14} content, the daily excretion of C^{14} as uric acid has been calculated. This information has been plotted cumulatively and expressed in percentage of administered C^{14} in Figure 2. In control subjects C. L. and W. W., 0.15 and 0.18 per cent of the C^{14} fed as carboxyl-labeled glycine appeared in urinary uric acid in eight days. In H. B., 0.064 per cent appeared in three days, an entirely normal value despite his first day maximal specific activity result. In contrast, the asymptomatic hyperuricemic subject, R. A., incorporated 0.29 per cent of the administered C^{14} into uric acid in seven days, and the patients with clinical gout incorporated from 0.29 to 0.66 per cent in six to seven days. Subject H. H. incorporated 0.18 per cent in four days, again a high value and consistent with results on the other gouty subjects. When the results are expressed in terms of cumulative incorporation of isotope over several days, it is clear that gouty subjects, as a group, differ from controls in the efficiency with which they utilize a tracer dose of glycine-1-C^{14} in the synthesis of uric acid. In the patients

[1] Data on control subject H. B., and gouty subject H. H., are not shown in Figures 1 or 2, since samples were collected for only three and four days, respectively.

studied, there was no overlap between gouty subjects and control subjects. The cumulative C^{14} incorporation into uric acid in the gouty patients exceeded that found in the controls by two- to five-fold.

It will be noted that the cumulative isotope incorporation curve of E. H., the gouty subject excreting 1,054 mg. of uric acid daily, did not differ greatly from those of the other gouty subjects, with the exception that during the first 24 hours following administration of glycine-C^{14} there was a greater incorporation of C^{14} into uric acid than in any other subject (this despite a specific activity value on Day 1 which was identical with that of control H. B.). The significance of this finding is not clear, however, particularly since an even greater rate of incorporation is found on Days 2 and 3 in patient W. R., who excreted only 313 mg. of uric acid daily. In this study, there was no apparent correlation between the basal excretion of uric acid and the per cent of ingested isotope appearing in urinary uric acid (*cf.*, Figure 2 and Table I).

There was also no clear relationship between the results obtained and the advent of an acute gouty attack. Patient W. R. was recuperating from an acute gouty attack which had had its onset four days prior to the administration of isotope, and V. L. developed an acute attack on the second day of the study and was treated with colchicine on Days 3 and 4. Nevertheless, the results procured in these subjects were similar to those of the other gouty subjects.

Labeling of expiratory CO_2

The labeling of expiratory CO_2 was determined following oral administration of glycine-1-C^{14} in gouty subject D. W. The specific activity of expired CO_2 was maximal 10 to 20 minutes following the administration of glycine. CO_2 collected at five hours still had significant C^{14} content, but samples collected at 24 and 48 hours did not measure significantly above background by the counting methods employed.

The peak specific activity of expired $C^{14}O_2$ was 2,605 c.p.m. per milliatom of carbon, calculated under conditions equivalent to those under which uric acid values were expressed. This value was some six-fold greater than the maximal specific activity of uric acid (396 c.p.m. mM), attained on the second day. Since CO_2 is known to be the precursor of C-6 of the purine ring (2, 15), the possibility was considered that C^{14} fed as glycine-1-C^{14} had labeled uric acid not only in C-4 as a consequence of incorporation of intact glycine, but also in C-6 via the bicarbonate pool. For this reason, the

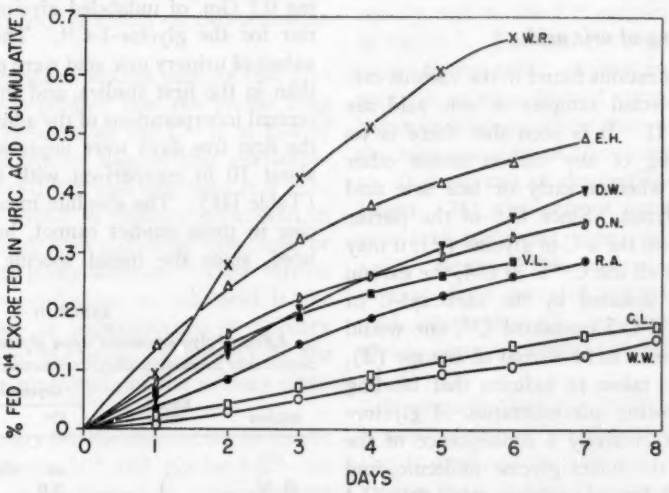

FIG. 2. THE CUMULATIVE EXCRETION OF C^{14} IN URINARY URIC ACID FOLLOWING ORAL ADMINISTRATION OF GLYCINE-1-C^{14}

The per cent of the administered glycine-1-C^{14} which has been excreted as uric acid-C^{14} has been plotted cumulatively.

TABLE II

Degradation of uric acid *

Subject	Diagnosis	Day	C-(4+5)†	Uric acid	C-(4+5) uric acid
			c.p.m./mM‡	*c.p.m./mM*	
W. W.	Control	1	285	297	0.96
		4	808	824	0.98
V. L.	Gout	1	1,330§	1,358	0.98
		5	573	594	0.96
D. W.	Gout	2	386	396	0.98
		5	320	335	0.96
D. D.‖	Myeloid metaplasia	3	866§	908	0.95
		12	802	798	1.00

* C-6 and C-(2+8) were also isolated on each sample and counted as $BaCO_3$.

† C-(4+5) were isolated and counted as glyoxylic acid semi-carbazone.

‡ These are actual counting values. Those plotted in Figure 1 have been normalized to correspond to a standard dose of five μc. of glycine-1-C^{14}.

§ These two samples were further degraded (2) so as to obtain C-4 and C-5 individually as $BaCO_3$. Virtually all the C^{14} was found in the $BaCO_3$ representing C-4 of the purine ring. Since separation of C-5 and C-4 is not complete (2), the small amount of C^{14} found in C-5 was regarded as contamination.

‖ D. D., a 42 year old woman with myeloid metaplasia, received 20 μc. of glycine-1-C^{14}. This study will be reported in detail elsewhere (8).

various carbon atoms of uric acid were isolated from selected samples and analyzed for their specific isotope contents.

Position of labeling of uric acid

The C^{14} concentrations found in the various carbon atoms of selected samples of uric acid are shown in Table II. It is seen that there is no significant labeling of any carbon atoms other than C-(4+5), whether early or late uric acid samples are analyzed. Since C-5 of the purine ring is derived from the α-C of glycine (2), it may be presumed that all the C^{14} is in C-4, the carbon atom specifically donated by the carboxyl-C of glycine (2, 3). If C-5 contained C^{14}, one would not expect C-(2+8) to be devoid of isotope (2). These results are taken to indicate that labeling of uric acid following administration of glycine-1-C^{14} is almost exclusively a consequence of the incorporation of the intact glycine molecule, and that secondary labeling of positions other than C-4 is negligible. These results are in striking contrast to those of Shemin and Rittenberg (1) and of Seegmiller, Laster, and Stetten (16), who found appreciable labeling of positions other than N-7 of uric acid after feeding N^{15}-glycine.

Effect of glycine carrier upon glycine-1-C^{14} utilization

In these studies, seven consecutive gouty subjects showed overincorporation of glycine-1-C^{14} into urinary uric acid, irrespective of the stage of their disease or of the magnitude of urinary uric acid excretion. Had these studies been conducted with glycine-N^{15}, one would have anticipated that perhaps all five subjects showing normal urinary urate excretions would have showed normal isotope incorporation. Indeed, subject O. N. was subsequently given glycine-N^{15} on two occasions and did show normal N^{15} incorporation values (17). The explanation cannot lie in the distribution of isotope in uric acid, for the non-specific labeling of uric acid by N^{15} would tend to introduce more, not less, N^{15} than C^{14}. A potential factor therefore was that of dose, since in studies with glycine-N^{15}, generally 0.1 Gm. per Kg. is given, whereas in the studies reported herein with glycine-1-C^{14}, doses of 0.2 to 2.0 mg. per patient were employed.

To test the influence of the dose factor the studies on O. N. and V. L., gouty subjects excreting normal quantities of urate, were repeated employing 0.1 Gm. of unlabeled glycine per Kg. as carrier for the glycine-1-C^{14}. The specific activity values of urinary uric acid were considerably lower than in the first studies, and the cumulative percentual incorporations of the administered C^{14} over the first five days were depressed by a factor of about 10 in comparison with the initial studies (Table III). The absolute incorporations of glycine in these studies cannot, however, be calculated, since the initial specific activities of the

TABLE III

Effect of glycine carrier upon glycine-1-C^{14} utilization

Subject	Experiment	Glycine fed		Uric acid-C^{14} formed in 5 days
		C^{14}	C^{12}	
		μc.	*Gm./Kg.*	*% administered C^{14}*
O. N.	I	5.0		0.29
	II	4.3	0.1	0.024
V. L.	I	10.0		0.26
	II	15.0	0.1	0.027

glycine pools functioning in purine synthesis (18) are unknown.

DISCUSSION

In an earlier study, Benedict, Yü, Bien, Gutman, and Stetten (4) found that control subjects and gouty subjects excreting normal quantities of uric acid incorporated 0.1 to 0.2 per cent of a test dose of N^{15}, fed as glycine, into uric acid in a nine-day period, whereas two gouty subjects excreting excessive quantities of uric acid incorporated much larger quantities of N^{15}. In the latter patients, 0.45 and 0.56 per cent, respectively, of the N^{15} appeared in urinary uric acid in nine days.

In the present study, the incorporation of C^{14}, fed as glycine-1-C^{14}, into uric acid was also of the order of 0.1 to 0.2 per cent in eight days for the control subjects and was 0.49 per cent in seven days in E. H., a gouty subject known to excrete large quantities of uric acid in urine. However, all five of the gouty subjects who exhibited normal basal uric acid excretions also showed high cumulative isotope incorporation values, which ranged from 0.29 to 0.66 per cent of the fed C^{14} in six to eight days. Some or all of these subjects might be anticipated to show normal incorporation values if studied by the glycine-N^{15} technique. Indeed, as mentioned above, one of them did show normal N^{15} incorporation values on two occasions (17).

The urate degradation studies indicate that glycine has been incorporated into the purine ring intact, and that virtually all the C^{14} is in C-4 of uric acid. These results suggest that overincorporation of glycine-1-C^{14} into uric acid is a consistent metabolic defect in primary gout, both in those with the gouty trait and those who have, or have had, clinical gouty arthritis. They further suggest that overproduction of uric acid is the fundamental cause of hyperuricemia in primary gout, regardless of the stage or severity of the disease or of the magnitude of the urinary urate excretion.

All of the reasons for the differences in results obtained with glycine-N^{15} and glycine-1-C^{14} are not clear. At least two factors do appear to have roles. First, the introduction of N^{15} into positions other than N-7 of the purine ring, because of transamination and entry of N^{15} into aspartic acid and the amide-N of glutamine, the precursors of N-1 and of N-3 and N-9, respectively (19–21), would tend to introduce a greater percentage of a given dose of glycine-N^{15} into uric acid than of glycine-1-C^{14}, which appears to label C-4 quite specifically. Second, the large dose of glycine required for the N^{15} studies appears to result in a decided reduction of the percentual incorporation of C^{14} into uric acid. These are opposing influences, and the apparent good agreement of results obtained with glycine-N^{15} and glycine-1-C^{14} in control subjects and in gouty subjects excreting large quantities of uric acid may therefore be fortuitous. The influence of the dose factor has not as yet been evaluated in control subjects or in gouty subjects excreting large quantities of urate, so a more definitive explanation of this problem is not now possible.

The curves of prompt and excessive enrichment of urinary uric acid found in the gouty subjects fed glycine-1-C^{14} resemble those published by Stetten's group for their gouty subjects who incorporated N^{15} from glycine excessively into uric acid. In explanation of these N^{15} curves, the arguments advanced by Benedict and associates (22) and by Stetten (23) for a shunt mechanism of urate synthesis, whereby dietary glycine can promptly enter urinary urate without the obligatory intervention of nucleic acid purines, apply equally well to the C^{14} enrichment curves shown in Figure 1. The configurations of the curves obtained in the gouty subjects indicate that labeled uric acid is being formed excessively and is turning over more promptly than normal. And since the turnover time of ribonucleic acids is about 8 to 10 days, and of desoxyribonucleic acids even longer (24), the prompt enrichment of urinary uric acid could not have proceeded via nucleic acids. Some of the mechanisms permitting this prompt direct entry of isotope into urinary uric acid, which obviously occurs to a limited extent also in normal subjects, are currently under study (8, 25).

If normally the direct pathways contribute only a relatively small fraction of the total uric acid that is produced per unit time (the larger portion arising by traditional reactions involving nucleic acid catabolism), it then becomes possible to offer an explanation as to why a two- to five-fold increment in utilization of a tracer dose of glycine-

1-C^{14} for urate synthesis in primary gout is not of necessity indicative of a two- to five-fold increment in total uric acid synthesis. Suppose, for example, that of a given quantity of purine nucleotides formed from glycine, 25 per cent is normally rapidly cleaved and converted to uric acid. A doubling of the rate of nucleotide synthesis, would then, in the absence of any change in the quantity of nucleotides utilized in nucleic acid production, result in a five-fold increase in the appearance of labeled glycine in uric acid while representing only a two-fold increment in total uric acid synthesis. Such a mechanism, operating variably in different patients, might explain the lack of a high urinary urate output in W. R. despite a very great C^{14} incorporation into uric acid, as contrasted with the very high urinary urate outputs in E. H. and H. H. associated, more logically, with high C^{14} incorporation. According to this concept, primary gout might involve a defect in the regulation of nucleotide synthesis and degradation, purine nucleotide generation in excess of tissue needs resulting in excessive uric acid production by direct routes of synthesis.

These studies do not, however, explain why gouty subjects who are overproducers of uric acid do not all excrete excessive quantities of uric acid in urine. Several factors may be of importance. It has been shown that the fraction of filtered urate reabsorbed by gouty persons is not significantly different from normal. This suggests that absence of overexcretion of urate in the presence of an elevated serum level of urate is a reflection of some degree of impairment of glomerular filtration, and several studies indicate that this may be the most common explanation (26–28). Impairment of renal function was demonstrated in W. R., and may have been present in mild degree in other subjects.

The uric acid not excreted via the kidney has three quantitatively important fates available to it: deposition in tophi, excretion by way of bile and gastrointestinal tract (29), and destruction in tissues (30, 31). Two of our patients (D. W. and V. L.) were observed to develop tophi about the time the studies were performed. Excretion by way of bile may be quite variable in magnitude (29), and recently it has been stated that in some patients whose body uric acid is increased, a greater *percentage* of uric acid is oxidized than in normals (32). Thus, compensating dispositions of urate may exist, and may condition the severity of the hyperuricemia. These factors require better definition, but the evidence available at this time suggests that overproduction of uric acid in primary gout is the fundamental defect responsible for the hyperuricemia of the disease.

SUMMARY

1. The rate of generation of uric acid in gouty subjects has been re-investigated in studies wherein tracer doses of glycine-1-C^{14} were fed and the excretion of C^{14} in urinary uric acid was determined.

2. The gouty subjects comprised six patients with histories of acute gouty arthritis, and one with the trait of asymptomatic hyperuricemia. Two gouty subjects excreted excessive quantities of urate in urine, whereas the other four, and the asymptomatic hyperuricemic subject, excreted quantities of urate well within the range of normal.

3. All seven gouty subjects exhibited quantitatively excessive incorporation of C^{14} into urinary urate. Degradation of the labeled urate suggested that glycine had been incorporated intact and indicated that non-specific labeling of the purine nucleus by secondary reactions was negligible.

4. These results constitute strong evidence that overproduction of uric acid from glycine and other small molecules is the fundamental defect responsible for the hyperuricemia of primary gout.

5. The characteristics of the curves of C^{14} incorporation into uric acid strongly suggest, as did earlier studies with glycine-N^{15}, that nucleic acids are not involved in the overproduction of uric acid in primary gout.

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URINARY EXCRETION OF ACID MUCOPOLYSACCHARIDES BY PATIENTS WITH RHEUMATOID ARTHRITIS [1]

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Several methods have been described for the determination of urinary acid mucopolysaccharides (1–4). It has been found that in normal individuals the daily urinary excretion varies with age and sex (4, 5). A few attempts have been made to correlate urinary excretion of acid mucopolysaccharides with different disease states. Craddock and Kerby (6) have reported that patients with impairment of renal function have a urinary excretion of acid mucopolysaccharides lower than that of normal individuals. They also found no significant differences between normal individuals and diabetic patients without renal involvement. In patients with rheumatoid arthritis Badin, Schubert, and Vouras (7) have demonstrated a high plasma concentration of acid mucopolysaccharides, but there have hitherto been no data available concerning their urinary excretion.

This paper presents data on the urinary excretion of acid mucopolysaccharides by patients with rheumatoid arthritis and discusses the identity of the urinary acid mucopolysaccharides excreted in this disease.

MATERIAL AND METHOD

The study was performed on eight patients hospitalized for active rheumatoid arthritis. All of them showed a moderately elevated erythrocyte sedimentation rate, low-grade fever, involvement of several joints, and typical radiographic alterations. All patients were untreated when the urine collection was started. Two of them, however (M.G. and P.C.), discontinued treatment a few days before the urine collection was started. Urinary acid mucopolysaccharides were determined according to the method described by Di Ferrante and Rich (4), by which the urinary acid mucopolysaccharides are precipitated, from an aliquot of the 24-hour urine, with cetyl-trimethyl-ammonium bromide. After removal of the precipitant the glucuronic acid content of the precipitate is determined. The results in Table I and in Figure 1 are expressed as mg. of glucuronic acid per 24 hours.

In five patients (P.C., B.E., E.E., W.J., and G.A.) the urinary excretion of acid mucopolysaccharides was measured both before and during periods of oral treatment with sodium salicylate.

In order to collect an amount of urinary acid mucopolysaccharides sufficient for purification and analysis, 20 liters of pooled urine from patients with rheumatoid arthritis were adjusted to pH 5 with 2 N HCl, and 8 Gm. of cetyl-trimethyl-ammonium bromide were added. After 24 hours at 0° C., the fine precipitate present was collected, by filtration under suction, on a thin layer of Hyflo super-cel. The cake of Hyflo super-cel was then washed in a 250-ml. centrifuge bottle with several 150-ml. aliquots of 95 per cent ethyl alcohol saturated in NaCl, until the supernatant obtained after centrifugation was colorless. The sediment was dried in a desiccator, under vacuum and in presence of paraffin chips, and then extracted three times with 30 ml. of distilled water. The aqueous extract was reprecipitated with three volumes of 95 per cent ethyl alcohol saturated in NaCl. After 24 hours at 0° C., the precipitate was collected by centrifugation, dissolved in 100 ml. of water and cleared of insoluble material by further centrifugation at 13,000 G for 30 minutes. To the clear solution obtained, NaCl was added to 0.58 M final concentration. This solution was left at 0° C. overnight and then centrifuged in a Spinco analytical centrifuge at 56,100 r. p. m. for 4 hours and 24 minutes (rotor J). The supernatant was electrodialyzed and brought to dryness.[3] It was dissolved again in 10 ml. of glycine-NaCl-HCl buffer pH 1.45, to which 5 mg. of crystallin pepsin [4] were added. The solution was incubated at 37° C. for 24 hours. At the end of this period, 15 ml. of phosphate buffer M/15, pH 7.5 and 10 mg. of crystallin trypsin [4] were added to the solution and incubation was resumed for an additional 24 hours. At the end of the incubation period, the solution was electrodialyzed and then filtered through a thin layer of Hyflo super-cel, under aspiration. The clear filtrate was brought to 50-ml. volume and then extracted three times with 20 ml. of 88 per cent phenol in water. The aqueous layer was lyophilized and the residue was dissolved in 10 ml. of water. After electrodialysis, the final volume was brought to 25 ml.

On aliquots of this solution the following determinations were performed:

[1] This work was supported by the Atomic Energy Commission.

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[3] As will be published elsewhere, the sediment thus obtained has been identified as the urinary mucoprotein reacting with various viruses, described by Tamm, Bugher, and Horsfall (8).

[4] Mann Research Laboratories, Inc., New York, N. Y.

1) Glucuronic acid, on four aliquots 0.2 ml. each according to the method of Dische (9), using glucurone as standard;
2) Nitrogen, on four aliquots 1 ml. each, according to the method described by Hiller, Plazin and Van Slyke (10);
3) A 5-ml. aliquot was hydrolyzed with 4 N HCl, in a sealed tube at 100° for six hours. After the acid was evaporated and the dry residue dissolved in a minimal amount of water, part of the hydrolysate was used for hexosamine determination, according to a modification of the Elson-Morgan method (11); part was used for the chromatographic differentiation between glucosamine and galactosamine, according to the technique described by Gardell (12), and part was used for paper chromatography according to Hausmann's technique (13) for the detection of amino acids. This chromatogram was dried at 100° for 10 minutes, then sprayed with a solution of 0.1 per cent ninhydrin in butanol containing 5 per cent collidine;
4) A 5-ml. aliquot was oxidized with concentrated HNO_3 in a sealed tube for eight hours at 180°. The acid was removed by evaporation in a desiccator over $CaCl_2$ and NaOH and the residue dissolved in redistilled water. On duplicate aliquots of this hydrolysate the sulfur was determined, as sulfate, by conductometric titration with a standard solution of $BaCl_2$, using a simple electric circuit, modified from that given by Shedlovsky (14);
5) Paper chromatography, according to the method of Kerby (15). The paper was stained with a solution of 0.1 per cent toluidine blue in 30 per cent ethyl alcohol and the excess of stain was removed

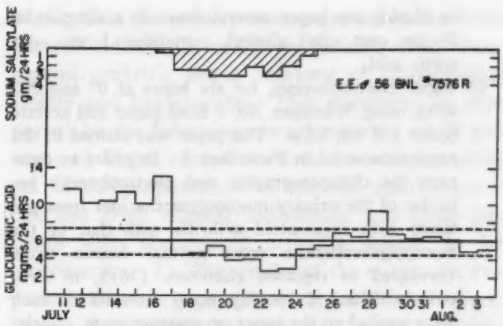

FIG. 1. INFLUENCE OF SODIUM SALICYLATE ADMINISTRATION ON URINARY EXCRETION OF ACID MUCOPOLYSACCHARIDES IN PATIENTS WITH RHEUMATOID ARTHRITIS

Mean value for normal men (————) and its range (- - - -) are also indicated.

Table I

Urinary excretion of acid mucopolysaccharides by patients with rheumatoid arthritis, before and during treatment

Patients	Treatment	Range mg/24 hr.	Average ± stand. dev. mg/24 hr.
P.C. ♀ 49	None (6 days)	5.1 - 6.2	5.8 ± .3
	Na Salicylate 4.5 gm/day (22 days)	2.5 - 6.6	4.2 ± .6
B.E. ♀ 39	None (5 days)	7.0 - 7.8	7.4 ± .3
	Na Salicylate 4.5 gm/day (12 days)	2.6 - 6.0	4.5 ± .6
E.E. ♂ 57	None (4 days)	10.0 - 13.0	11.0 ± .7
	Na Salicylate 2.4 gm/day (9 days)	2.7 - 5.4	3.9 ± .6
W.J. ♂ 34	None (6 days)	6.5 - 9.4	8.1 ± .8
	Na Salicylate 4.5 gm/day (9 days)	3.3 - 5.6	4.1 ± .4
G.A. ♂ 55	None (11 days)	9.4 - 14.6	11.2 ± .9
	Na Salicylate 6.3 gm/day (13 days)	4.0 - 9.4	5.6 ± .9
W.W. ♂ 72	None (5 days)	8.9 - 9.8	9.3 ± .3
H.F. ♂ 56	None (5 days)	10.6 - 12.8	11.9 ± .6
M.G. ♂ 46	None (14 days)	4.7 - 10.0	6.6 ± .9
Normal Subjects			
Women		2.7 - 5.4	3.7 ± .2
Men		4.4 - 7.2	6.0 ± .3

by rinsing the paper several times in a solution of 30 per cent ethyl alcohol, containing 1 per cent acetic acid;

6) Paper electrophoresis, for six hours at 0° and 200 volts, using Whatman No. 3 filter paper and acetate buffer pH 4.6, 0.1 μ. The paper was stained in the same manner as in Procedure 5. In order to compare the chromatographic and electrophoretic behavior of the urinary mucopolysaccharides from patients with rheumatoid arthritis with that of the mucopolysaccharides from normal human urine (prepared as reported elsewhere (16)), in Procedures 5 and 6 roughly equal amounts of each were applied on the paper on separate spots. Skeletal chondroitinsulfate and hyaluronate from human umbilical cords [5] were also applied on the paper as reference standards;

7) Incubation with bacterial hyaluronidase. Two hundred micrograms of acid mucopolysaccharides from urine of patients with rheumatoid arthritis and 200 micrograms of acid mucopolysaccharides from normal human urine were incubated, in duplicate, with 100 turbidity reducing units of bacterial hyaluronidase.[6] The extent of depolymerization of the two substrates after 30 minutes of incubation was measured using the turbidimetric method described by Di Ferrante (17).

RESULTS

Table I summarizes the values obtained in patients with rheumatoid arthritis, before and during treatment with sodium salicylate. The normal values for women and men, as determined with the same method (4), are also included. The differences between the values of untreated patients with rheumatoid arthritis and the values of normal individuals are statistically significant ($p < 0.01$), except in patient M.G. In the five patients treated with salicylate, the fall in the mucopolysaccharides excretion during treatment is statistically significant (p of difference < 0.01).

Figure 1 shows in detail the urinary excretion of acid mucopolysaccharides in patient E.E. before, during, and after sodium salicylate administration.

The possibility that some dialyzable material, containing glucuronic acid, could be partially responsible for the color obtained with the carbazole reagent and that the effect of salicylate could be due to changes in such component, was investigated. Extensive dialysis of solutions of the urinary material precipitated with cetyl-trimethylammonium bromide demonstrated the absence of dialyzable material able to develop color with the carbazole reagent, either before or during treatment with salicylate.

To investigate the possibility that products of excretion of sodium salicylate could interfere with the reaction between acid mucopolysaccharides and the precipitant, a recovery test was conducted, adding 1 mg. and 0.1 mg. of skeletal chondroitinsulfate to duplicate 30-ml. aliquots of urine obtained from patients under salicylate treatment. The recovery ranged between 94 and 104 per cent.

In Table II are presented the molar ratios of nitrogen, sulfur, glucuronic acid and glucosamine to galactosamine, for the mucopolysaccharides obtained from normal human urine and for those obtained from urine of patients with rheumatoid arthritis.

[5] Worthington Biochemical Sales Company, Freehold, New Jersey.

[6] Obtained through the kindness of Dr. K. W. Thompson, Organon Inc., Orange, New Jersey.

Table II

Molar ratios of some constituents of mucopolysaccharides from normal human urine and from urine of patients with rheumatoid arthritis

Material	Nitrogen‡	Sulfur‡	Glucuronic acid	Galactosamine(*)	Glucosamine
Mucopolysaccharides from normal human urine	1.75	1.10	1.27	1.00	0.10
Mucopolysaccharides from urine of patients with rheumatoid arthritis	3.57	1.19	1.83	1.00	0.53

(*)Galactosamine is arbitrarily assigned a value of 1.00.

‡ Nitrogen and sulfur figures are atoms N and S per molecule of galactosamine.

The paper chromatogram of the hydrolyzed urinary mucopolysaccharides from patients with rheumatoid arthritis, developed according to Hausmann's technique and stained with ninhydrin, revealed, besides a major component having the same mobility as a standard of glucosamine and galactosamine hydrochloride,[7] at least 10 additional components. On paper chromatography, according to Kerby (15), and on paper electrophoresis, the urinary mucopolysaccharides from normal individuals and from patients with rheumatoid arthritis presented a similar pattern, namely a fast-moving component having a mobility similar to that of a standard of skeletal chondroitin-sulfate, and another component, migrating at a rather slower rate, with a mobility similar to that of a standard of hyaluronate. Incubation for 30 minutes with 100 turbidity reducing units of bacterial hyaluronidase produced 30 per cent depolymerization of the preparation obtained from patients with rheumatoid arthritis and 15 per cent depolymerization of the preparation obtained from normal individuals. Incubation under the same conditions, for intervals of time shorter or longer than 30 minutes, demonstrated that the last interval allowed maximal depolymerization of the substrates.

DISCUSSION

The presence in normal human urine of an acid mucopolysaccharide similar to skeletal chondroitinsulfuric acid has been demonstrated (16). Both hyaluronic acid and chondroitinsulfuric acid have been found in normal human plasma (18). Recently, hyaluronic acid has been isolated in a relatively large amount from the sera of two patients with reticulum cell sarcoma and neuroblastoma (19). Considering that urinary acid mucopolysaccharides are present in urine obtained from the renal pelvis (5) and that their average molecular weight is approximately 10,000[8] (16), it appears correct to assume that the urinary material derives from the similar one present in plasma.

The analytical data of Table II suggest the possibility that the urinary mucopolysaccharides from normal individuals and from patients with rheumatoid arthritis are a mixture of chondroitinsulfate and hyaluronate. This possibility is supported by the following considerations: 1) 10 per cent and 35 per cent of the total hexosamine present in the preparations of urinary mucopolysaccharides from normal individuals and from patients with rheumatoid arthritis appear to be glucosamine. 2) The results of the enzymatic experiments are in good agreement with these values. In fact, incubation of aliquots of the two preparations with bacterial hyaluronidase produced respectively 15 per cent and 30 per cent depolymerization of the substrates. 3) The sulfur and the galactosamine of the two preparations are approximately in equimolar ratio. The excess of sulfur may be due to sulfur-containing amino acids from protein contaminants.

Is the different ratio chondroitinsulfate/hyaluronate in the two groups of individuals merely a consequence of the different techniques used for the purification of the urinary materials, or is it a reflection of some difference in the metabolism and/or excretion of acid mucopolysaccharides in the two groups? Analysis of several preparations of acid mucopolysaccharides obtained from normal human urine using the technique described here has demonstrated that approximately 30 per cent of the hexosamine present in them is glucosamine. Therefore, it seems correct to assume that the difference in hyaluronate content of the two preparations presented in Table II is due to the different techniques used for their purification.

The administration of sodium salicylate to patients with rheumatoid arthritis appears to reduce the previously elevated urinary excretion of acid mucopolysaccharides. No data are available on the effect of this drug on the plasma level of acid mucopolysaccharides in similar conditions. It has been reported, however, that the administration of cortisone,[9] aspirin and gold, either together or separately, to patients with rheumatoid arthritis, reduces the elevated serum level of glycoproteins (20).

Several observations seem to prove that sodium salicylate decreases the uptake of sulfate-S^{35} by slices of cartilage *in vitro* (21) and the hexosamine content of the skin *in vivo* (22), effects which have

[7] With the solvent systems used, glucosamine and galactosamine are not distinguished.

[8] The average molecular weight of 10,000 was obtained for a preparation of urinary acid mucopolysaccharides previously incubated with trypsin and papain. An average value of 17,000 was obtained for a similar preparation not treated with proteolytic enzymes.

[9] Brand name of 17-hydroxy-11-dehydrocorticosterone.

been interpreted as indicative of inhibition in the synthesis of acid mucopolysaccharides produced by this drug. At present, however, it is not possible to state whether a decreased rate of synthesis is responsible for the diminution in the urinary excretion of acid mucopolysaccharides which follows the administration of salicylate to patients with rheumatoid arthritis.

SUMMARY

In patients with active, untreated rheumatoid arthritis the daily urinary excretion of acid mucopolysaccharides was higher than in normal individuals.

The administration of sodium salicylate to those patients decreased the urinary excretion of acid mucopolysaccharides.

Paper chromatography and paper electrophoresis, analytical and enzymatic data seem to indicate that the urinary acid mucopolysaccharides from normal individuals and those from patients with rheumatoid arthritis are a mixture of chondroitinsulfate and hyaluronate.

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STUDIES OF INTESTINAL DIGESTION AND ABSORPTION IN THE HUMAN [1]

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Our knowledge of the processes underlying the normal digestion and absorption in the human small intestine is incomplete in spite of a considerable volume of research devoted to this subject (1–3).

While balance studies and blood analysis of absorbed material only give indirect information in this respect, more definite knowledge of the digestion and absorption processes must include studies of the composition of the intestinal content of different parts of the intestine during digestion and absorption. Various techniques using intubation of the human intestinal tract (4, 5) have also provided results in this direction and have given some information about factors such as pH (6, 7), enzyme concentrations (6), and composition of ingested foodstuffs (8). Such information *per se*, however, is incomplete if it does not make it possible to calculate the extent of absorption at different levels. Such quantitative data have hitherto been obtained in special cases only by collecting intestinal content from segments isolated between balloons or proximal to occluding balloons (3, 9, 10) with techniques which are not generally applicable and which in many cases are physiologically unsuitable.

In the study presented here we have aimed at an analysis of the digestion and absorption processes in the small intestine of normal adult human subjects. Sampling at different levels of the intestinal tract after feeding a well-balanced liquid test meal has been effected with the transintestinal intubation technique as described by Blankenhorn, Hirsch, and Ahrens (5). This technique, which utilizes intubation with a polyvinyl tubing (inner diameter, 2.1 mm.), allows sampling at any desired level of the gut under conditions very closely resembling the physiological state. To get quantitative figures as to the sites and extent of absorption of various substances included in the test meal, a reference substance, polyethylenglycol, with a molecular weight of 3000 to 3700, has been incorporated into the test meal. Although highly soluble in water, this substance is not absorbed or decomposed in the intestinal tract.

It has thus been possible to follow the dilution of the meal at different levels and to follow the absorption of the various components of the foodstuffs over the length of the human small intestine. Simultaneous analysis of the intestinal content as to concentration of foodstuffs and their digestion product, different bile constituents, enzymes, and pH has given information about the milieu of digestion from which the absorption takes place in the normal human being.

MATERIAL AND METHODS

The material includes experiments on six human subjects between 16 and 40 years of age: two female and four males, without any known gastrointestinal disorders.

The subjects were intubated according to the principles of Blankenhorn, Hirsch, and Ahrens (5) using a polyvinyl tubing (inner diameter, 2.1 mm.),[2] and sampling was generally started when the tube had passed the entire gastrointestinal tract. Sampling was generally attempted from both the oral and anal end of the tubing from perforations on the tubing located 50 to 100 cm. apart and separated by a closed segment. In some cases sampling from the anal end was difficult or impossible since no free flow could be obtained. Altogether 16 successful experiments were performed on these subjects comprising 13 oral and 8 anal samplings. The test meal was usually given in the morning and intestinal content collected over a four-hour period. When the upper sampling was made from the duodenum or the first part of the jejunum two half-hour samples were taken during the first hour; later on and at lower levels hourly samples were taken. Sampling at levels lower than 300 cm. from the nose was only successful in one case.

When it appeared from the studies of these six subjects that the major site of the absorption of the food-

[1] This investigation was generously supported by the Swedish Medical Research Council.

[2] The tubing was generously supplied by the Pharmaseal Laboratories, Glendale, California.

stuffs was the distal duodenum and the first part of the jejunum six additional normal adults were studied. These subjects were intubated in the morning and given the test meal when the locus of aspiration had reached 70 to 125 cm. from the nose. In these cases 10-minute samples were taken during the first hour after the test meal and thereafter hourly samples were taken.

Usually the tubing moved slowly downwards during the sampling period and the distance from the nose to the site of aspiration for each sample is given as the mean value between the position at the beginning and the end of the sampling period. The position of the sampling area over the length of the intestine is given in cm. from the nose. To localize this level, X-rays were taken in some cases after injection of radio-opaque solution into the tubing. No attempts were made to localize exactly the position of the pylorus, ligament of Treitz, or the ileocecal valve. The variations in the position of these points have recently been reported by Hirsch, Ahrens, and Blankenhorn (11), and the arrows on the figures are calculated from their figures. They found the distance from nose to pylorus to be around 65 cm.; from nose to ligament of Treitz to be around 90 cm.; and from nose to ileocecal valve to be around 350 cm.

The test subjects were sitting in a chair during the collection period and the samples were collected in flasks chilled with crushed ice. At the beginning of the experiment the tubing was filled with water and flow was maintained by siphonage with the end of the tubing at floor level.

Test meal. The test meal given was a balanced liquid formula essentially with the composition given by Ahrens, Dole, and Blankenhorn (12). It consisted of a homogenized mixture of fat, protein, and carbohydrate, the distribution of calories being 40, 15, and 45, respectively. Included in the test meal is also polyethylenglycol (PEG), serving as an indicator of the test meal, and radioiodinated human serum albumin (RIHSA) serving as an indicator of the food protein. The composition of the test meal is given in Table I. Of this liquid formula 500 ml. was taken orally during the first 10 to 15 minutes of each experiment. The caloric content of the test meal is 1.25 calories per ml.

TABLE I

COMPOSITION OF TESTMEAL

	g	FAT	CARBOHYDRATE	PROTEIN
CORN OIL	74	74	–	–
SKIM MILK POWDER	126	0,5	50	63
DEXTROSE	138	–	138	–
PEG	5	–	–	–
RIHSA	1	~5μC I^{131}		1
WATER	1000	74,5	188	64
	mg per ml: 60		150	50

Methods of analysis. Polyethylenglycol (PEG)[3] concentrations were determined essentially according to Hydén (13).

Non-phospholipid fat was determined by weight after extraction of 5 to 10 ml. of the samples (acidified with hydrochloric acid) with a system containing equal parts of ethanol, diethyl ether, heptane and water as described by Ahrens and Borgström (8). In this way neutral fat and free fatty acids are obtained free from bile acids, amino acids, carbohydrate and salt. Less then five per cent of the total phospholipids present in the intestinal content is extracted with this method.

Lipid phosphorous was determined after extraction with 10 volumes of a mixture containing ethanol and acetone in equal volumes according to the Fiske and Subbarow method as used by Brante (14).

Free fatty acids were determined on the lipid extract by titration with 0.1-N NaOH using thymol blue as indicator.

Bile acids were determined with the method described by Sjövall (15). Optical density at 400 mμ was measured in a 1-cm. cell on the supernatant after precipitation of the proteins with ten volumes of ethanol.

Glucose in the intestinal content was determined by a modification of the method described by Sumner (16) using 3,5-dinitrosalicylic acid as reagent.

Amylase was determined by the method of Meyer, Noelting, and Bernfeld (17). The amylase activity is expressed as milligrams of maltose liberated in three minutes by 1 ml. of the sample at 25° C.

Sucrase activity was assayed according to a modification of the method of Sumner (18); the activity is given as milligrams of invert sugar produced in one hour by 1 ml. of the sample at 25° C.

Lipase was assayed with a spectrophotometric method using a triolein emulsion as substrate (19).

Trypsin and chymotrypsin were determined spectrophotometrically with a modification of the method described by Schwert and Takenaka (20), using bensoylarginine ethyl ester and tyrosine ethyl ester, respectively, as substrates (21).

Assay of I^{131}-activity was made in a well-type scintillation counter, 10^4 counts were counted on each sample. To get an estimation of the extent of hydrolysis of the protein we have determined the I^{131}-activity in the supernatant after phosphotungstic acid precipitation of the intestinal content (equal volumes of intestinal content, 5 per cent phosphotungstic acid and water) (22). One subject was fed a test meal containing approximately 10 microcuries ^{14}C-triolein. The ^{14}C activity was determined on the total fatty acids of each fraction with a conventional flow counter.

The percentage absorption of fat, carbohydrate and protein at a given level was calculated from the relation of the amount of these substances to PEG concentration in the test meal and the same relationship in

[3] The polyethylenglycol was obtained from Mo and Domsjö A.B., Sweden, under the name of "Polyglykol A 4000."

the collected samples. In the case of protein, the total amount of radioactivity in the intestinal content was considered to be protein bound in these calculations. *pH-values* were determined with a glass electrode as soon as possible after the collection of the samples. In some cases continuous recording of pH was undertaken with the electrodes built into a chamber of 1.5 ml. volume through which the intestinal content passed. The I^{131}-labelled human serum albumin (RIHSA) was purchased from The Radiochemical Centre, Amersham, England.

RESULTS AND DISCUSSION

The use of PEG as a reference substance

In 1953, Sperber, Hydén, and Ekman (23) reported the use of PEG as a reference substance in digestion studies. They used it in a study of ruminant digestion in the sheep and found it not to be absorbed or destroyed to any considerable extent in the alimentary tract and could recover more than 90 per cent in the feces. The behavior of different polyethylenglycols in the animal and human body had earlier been extensively studied by Shaffer, Critchfield, and Nair (24, 25) in connection with the introduction of these substances for pharmaceutical use (carbowax compounds). These authors (24) concluded that the higher polyethylenglycols are not absorbed after oral dosage in rat or man. Solid polyethylenglycols have also been reported suitable for use as reference substance for the estimation of fecal output in the cow (26).

The solid polyethylenglycols with a molecular weight of 3000 to 3700 used by Sperber, Hydén, and Ekman (23) and Corbett, Miller, Clarke, and Florence (26), therefore, seemed to be well documented for use as reference substance in studies of the digestion and absorption of water soluble substances in the human.[4] Replicate samples taken from the same freshly drawn intestinal content gave constant ratios of fat to PEG. It, therefore, seems justifiable to use the watersoluble PEG as a reference substance also for fat when the latter is present in the test meal in a finely emulsified form.

Recovery of test meal from the tube

The sampling technique used in this investigation does not aim at a complete recovery of the intestinal content that passes a certain level. The amount of test meal contained in each sample obtained from the tubing can instead be calculated from the concentration therein of the reference substance (PEG) included in the test meal. If the samples obtained through the nasal end of the tubing from the six persons subjected to transintestinal intubation are considered, a fairly constant proportion of the test meal, as a mean 22.6 ± 2.2 per cent, passed out through the tube during the four-hour sampling period. These figures include samples collected on levels from the duodenum down to 215 cm. from the nose. The distribution of the test meal recovered from the tube, as calculated from the PEG values over the different hourly collection periods in the above experiments, was found to be 29.5, 35.0, 23.8, and 11.7 per cent of the total amount recovered over the four-hour periods. Thus, the 500 ml. of test meal fed to these subjects are delivered from the stomach over a four-hour period, with the largest portion during the second hour. At levels lower than 150 cm. from the nose (distal jejunum), the first hour samples usually did not contain any appreciable amount of test meal, indicating that the passage of the meal down the intestine is rather slow normally. When sampling from the duodenum and the first part of the jejunum, we generally observed that the flow ceased during the fourth hour, indicating that the stomach was empty at that time.

Concentration in intestinal content of PEG

Figure 1 shows the concentration of the reference substance in the samples collected at different levels. The general trend is a three- to fivefold dilution of the test meal in the duodenum followed by a concentration further down the small intestine. At levels below 150 cm. from the nose we will find both very high and very low figures. The reason for the large differences found at these low levels will probably be found in the fact that the recovery through the tube of samples containing test meal is more by chance due to the small volume of viscous content passing. In some cases very low PEG values are found in samples obviously mostly representing intestinal secretion.

From the PEG values in the duodenum it can be calculated that the 500-ml. test meal during its pas-

[4] In two experiments a patient with ileostomy after total colectomy was fed the test meal and 103 and 95 per cent of the PEG recovered in the intestinal contents collected for 48 hours.

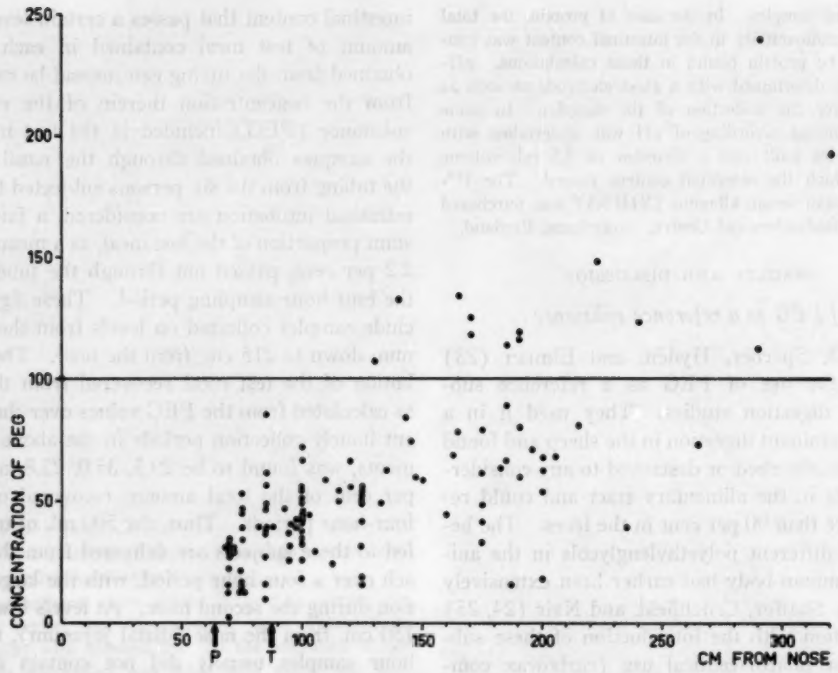

FIG. 1. CONCENTRATION OF POLYETHYLENGLYCOL (PEG) IN INTESTINAL CONTENT COLLECTED AT DIFFERENT LEVELS OF THE SMALL INTESTINE AFTER FEEDING 500-GM. TEST MEAL
Concentration of PEG in the test meal equals 100.

sage through the duodenum is diluted to a volume of 1,500 to 2,500 ml.

Absorption of fat, glucose and protein

The presence in the test meal of a substance that is not absorbed nor digested makes it possible for the first time to calculate the absorption curves over the length of the human small intestine of the foodstuffs included in the meal. These curves for fat, glucose and protein are seen in Figure 2. It is seen that the absorption of these substances begins already in the distal part of the duodenum and is generally completed in the first 50 to 100 cm. of the jejunum, a little higher up for fat than for glucose and protein. In the lower part of the small intestine the glucose concentration reaches zero while there are always some substances present in the intestinal content at all levels which are extractable with fat solvents. That this fat is mainly of dietary origin is shown from the experiment in which ^{14}C-triolein was included in the fat of the test meal. In this subject intestinal content was collected at a level of 190 to 200 cm. from the nose during a four-hour period following the test meal. The lipid content of the four hourly samples was 5.1, 3.0, 6.9 and 3.1 mg. per ml. intestinal content with specific activities of 110, 71, 94 and 79 per cent of that of the fat of the test meal.

Studies of the digestion and absorption of proteins are complicated by the difficulty of differentiating between food protein and the rather large quantities of endogeneous protein contained in the secretions to the intestinal tract (27). To indicate the food protein we have added radioiodinated human serum albumin (RIHSA) to the test meal. Using filter-paper chromatography we have found that in various samples of intestinal content collected in connection with a test meal containing RIHSA less than 10 per cent of the I^{131} activity is in the form of inorganic iodine. Furthermore, Lavik, Mattews, Buckaloo, Lemm, Spector, and Friedell (28) have found that at the most 12 per cent of I^{131} bound to casein was liberated as

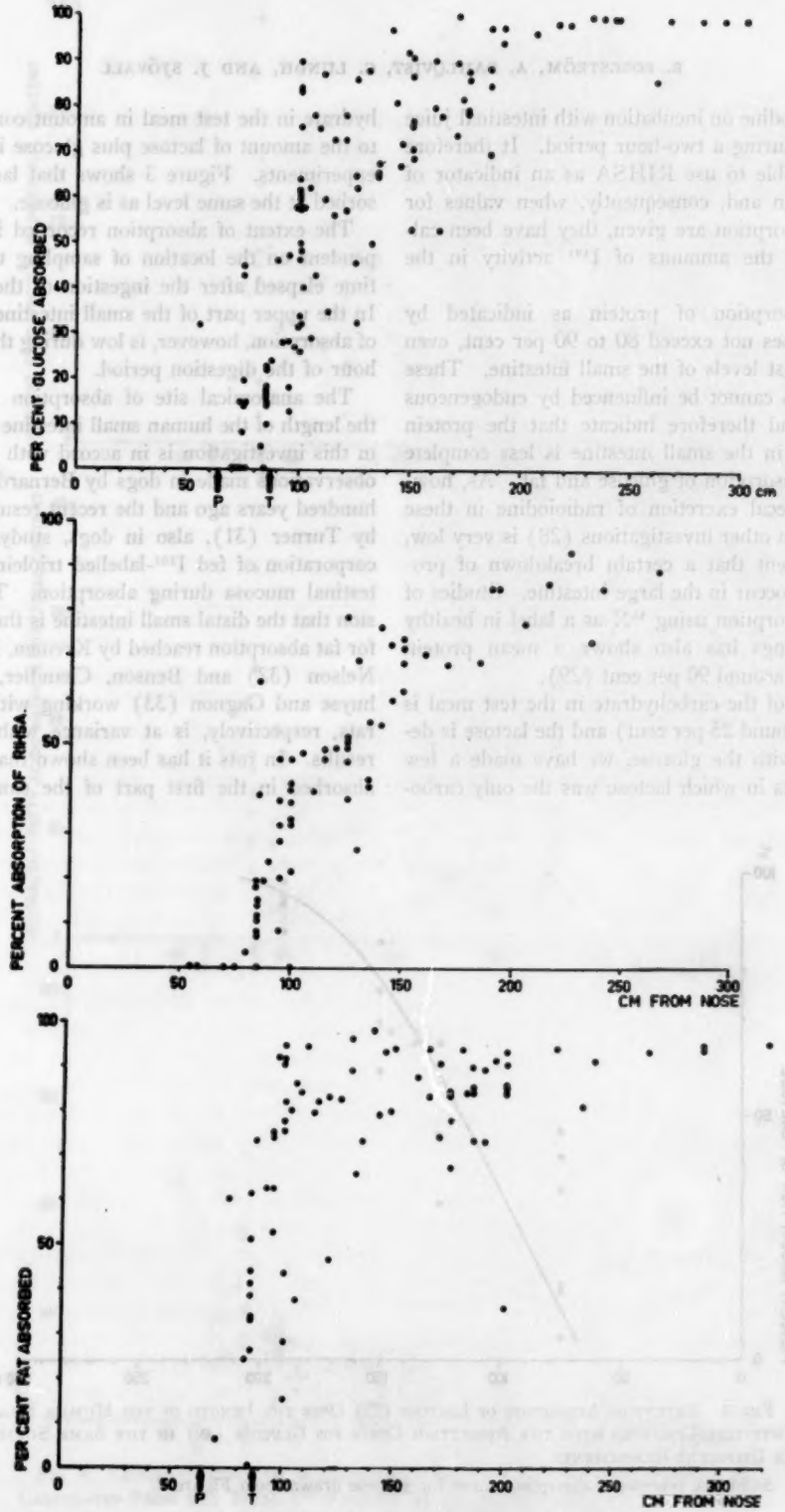

FIG. 2. ABSORPTION CURVES FOR FAT, GLUCOSE (PLUS LACTOSE) AND PROTEIN AS INDICATED BY I^{131} OVER THE LENGTH OF THE HUMAN SMALL INTESTINE

inorganic iodine on incubation with intestinal juice from dog during a two-hour period. It therefore seems feasible to use RIHSA as an indicator of food protein and, consequently, when values for protein absorption are given, they have been calculated on the amounts of I^{131} activity in the sample.

The absorption of protein as indicated by RIHSA does not exceed 80 to 90 per cent, even at the lowest levels of the small intestine. These calculations cannot be influenced by endogeneous proteins and therefore indicate that the protein absorption in the small intestine is less complete than the absorption of glucose and fat. As, however, the fecal excretion of radioiodine in these cases and in other investigations (28) is very low, it is apparent that a certain breakdown of proteins must occur in the large intestine. Studies of protein absorption using ^{15}N as a label in healthy human beings has also shown a mean protein absorption around 90 per cent (29).

As part of the carbohydrate in the test meal is lactose (around 25 per cent) and the lactose is determined with the glucose, we have made a few experiments in which lactose was the only carbohydrate in the test meal in amount corresponding to the amount of lactose plus glucose in the other experiments. Figure 3 shows that lactose is absorbed at the same level as is glucose.

The extent of absorption recorded is more dependent on the location of sampling than on the time elapsed after the ingestion of the test meal. In the upper part of the small intestine the extent of absorption, however, is low during the first half-hour of the digestion period.

The anatomical site of absorption of fat over the length of the human small intestine determined in this investigation is in accord with the general observations made in dogs by Bernard (30) one-hundred years ago and the recent results obtained by Turner (31), also in dogs, studying the incorporation of fed I^{131}-labelled triolein in the intestinal mucosa during absorption. The conclusion that the distal small intestine is the major site for fat absorption reached by Kremen, Linner, and Nelson (32) and Benson, Chandler, Vansteenhuyse and Gagnon (33) working with dogs and rats, respectively, is at variance with the above results. In rats it has been shown that glucose is absorbed in the first part of the small intestine

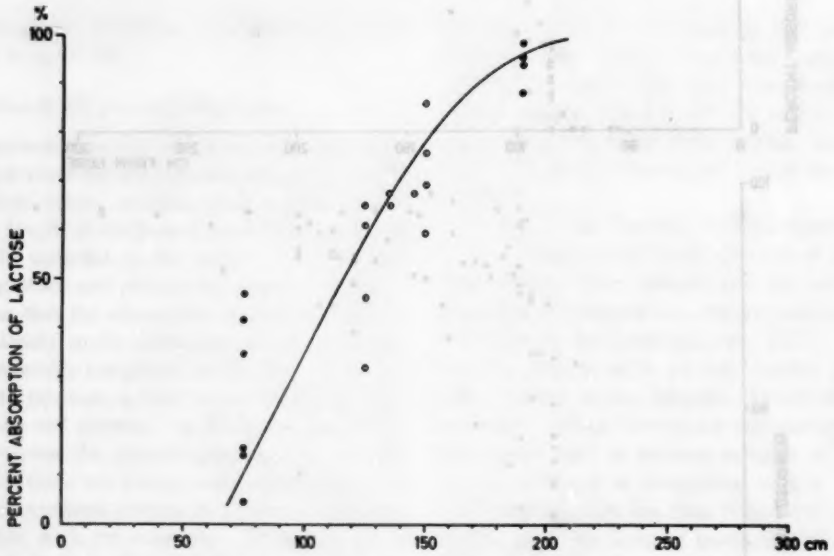

FIG. 3. EXTENT OF ABSORPTION OF LACTOSE (○) OVER THE LENGTH OF THE HUMAN SMALL INTESTINE COMPARED WITH THE ABSORPTION CURVE FOR GLUCOSE (●) IN THE SAME SUBJECT IN DIFFERENT EXPERIMENTS

Solid line represents absorption curve for glucose drawn from Figure 2.

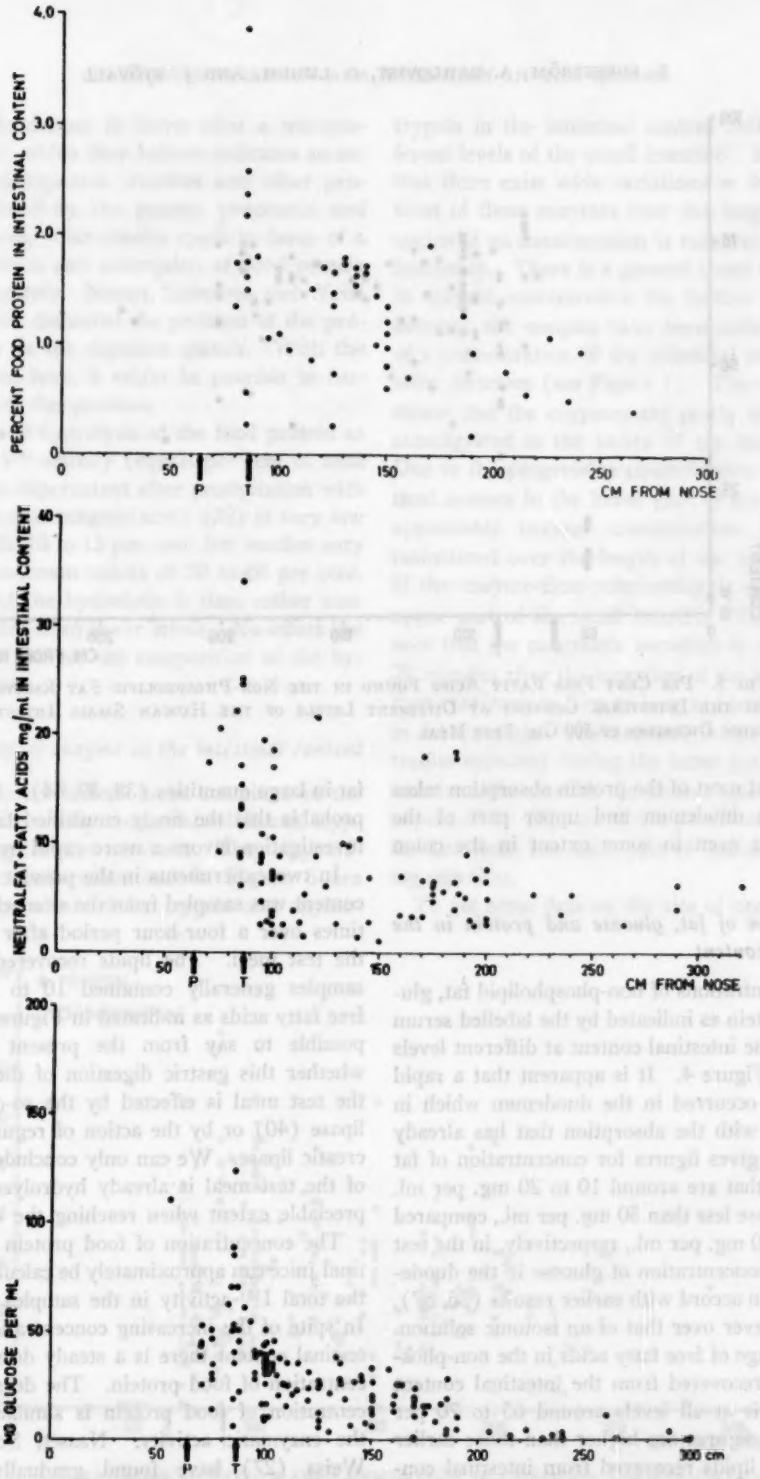

FIG. 4. CONCENTRATIONS OF NON-PHOSPHOLIPID FAT, GLUCOSE AND PROTEIN AS CALCULATED FROM THE TOTAL I^{131} ACTIVITY AT DIFFERENT LEVELS OF THE HUMAN SMALL INTESTINE AFTER FEEDING OF 500-GM. TEST MEAL

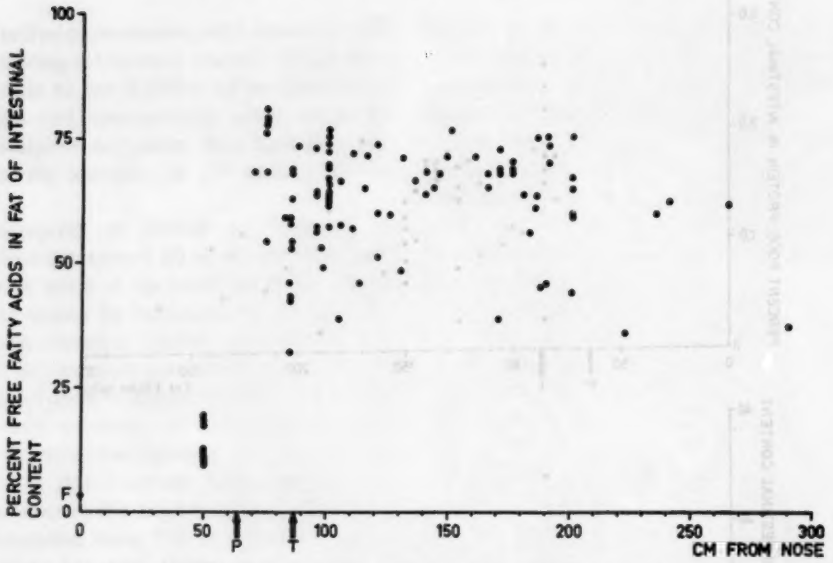

FIG. 5. PER CENT FREE FATTY ACIDS FOUND IN THE NON-PHOSPHOLIPID FAT RECOVERED FROM THE INTESTINAL CONTENT AT DIFFERENT LEVELS OF THE HUMAN SMALL INTESTINE DURING DIGESTION OF 500-GM. TEST MEAL

(34) and that most of the protein absorption takes place in the duodenum and upper part of the jejunum, but even to some extent in the colon (35).

Concentration of fat, glucose and protein in the intestinal content

The concentrations of non-phospholipid fat, glucose and protein as indicated by the labelled serum albumin in the intestinal content at different levels are seen in Figure 4. It is apparent that a rapid dilution has occurred in the duodenum which in combination with the absorption that has already started here gives figures for concentration of fat and protein that are around 10 to 20 mg. per ml. and for glucose less than 50 mg. per ml., compared to 60 and 150 mg. per ml., respectively, in the test meal. The concentration of glucose in the duodenum is thus in accord with earlier results (36, 37), practically never over that of an isotonic solution. The percentage of free fatty acids in the non-phospholipid fat recovered from the intestinal content (Figure 5) is at all levels around 65 to 70 per cent. These figures are higher than those earlier reported for lipids recovered from intestinal contents from animals and humans fed non-emulsified fat in large quantities (38, 39, 44). It seems very probable that the finely emulsified fat fed in this investigation favors a more rapid hydrolysis.

In two experiments in the present investigation content was sampled from the stomach at different times over a four-hour period after ingestion of the test meal. The lipids recovered from these samples generally contained 10 to 20 per cent free fatty acids as indicated in Figure 5. It is not possible to say from the present experiments whether this gastric digestion of the corn oil of the test meal is effected by the so-called gastric lipase (40) or by the action of regurgitated pancreatic lipase. We can only conclude that the fat of the test meal is already hydrolyzed to an appreciable extent when reaching the duodenum.

The concentration of food protein in the intestinal juice can approximately be calculated through the total I^{131}-activity in the samples (Figure 4). In spite of the increasing concentration of the intestinal content there is a steady decrease in concentration of food protein. The decrease in concentration of food protein is similar to that for the enzymatic activity. Nasset, Schwartz, and Weiss (27) have found gradually increasing amounts of total nitrogen in intestinal content of

dogs from duodenum to ileum after a non-protein test meal, which they believe indicates an accumulation of digestive enzymes and other proteins contributed by the gastric, pancreatic and intestinal juices. Our results speak in favor of a parallel digestion and absorption of food protein and enzyme protein. Nasset, Schwartz, and Weiss (27) also have discussed the problem of the protein turnover in the digestive glands. With the technique used here, it might be possible to further investigate this problem.

The extent of hydrolysis of the food protein as indicated by I^{131}-activity (equals per cent of total activity in the supernatant after precipitation with 5 per cent phosphotungstic acid) (22) is very low in the stomach, 10 to 15 per cent, but reaches very rapidly in duodenum values of 50 to 60 per cent. This extent of the hydrolysis is then rather constant in samples from lower levels. No effort has been made to analyze the composition of the hydrolyzed protein.

Concentrations of enzyme in the intestinal content

Of the enzymes known to be contained in the pancreatic juice we have determined lipase, trypsin, chymotrypsin and amylase. They approximately follow each other and in Figure 6 are seen the values obtained for trypsin and chymotrypsin in the intestinal content collected at different levels of the small intestine. It is apparent that there exist wide variations in the concentrations of these enzymes over the length of the intestine if no consideration is taken to the time relationship. There is a general trend to a decrease in enzyme concentration the further down in the intestine the samples have been collected in spite of a concentration of the intestinal content in the same direction (see Figure 1). This fact must indicate that the enzymes are partly inactivated or autodigested in the lumen of the small intestine. Due to the progressive concentration of the intestinal content in the lower part of the intestine an appreciable enzyme concentration, however, is maintained over the length of the small intestine. If the enzyme-time relationship is studied in the upper part of the small intestine (Figure 7) it is seen that the pancreatic secretion is started 10 to 20 minutes after the ingestion of the test meal and then continues to flow as long as there is any food in the stomach. The decrease in enzyme concentration apparent during the latter part of the first hour and the most of the second hour is probably explained by dilution of the pancreatic juice with the test meal, the main part of which passes during this time.

To get some data on the site of production and

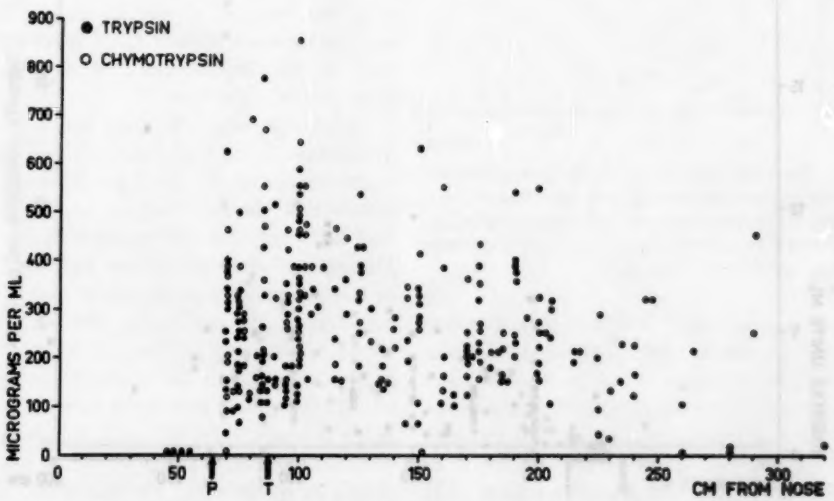

FIG. 6. CONCENTRATION OF TRYPSIN AND CHYMOTRYPSIN IN INTESTINAL CONTENT COLLECTED AT DIFFERENT LEVELS OF THE HUMAN SMALL INTESTINE AFTER 500-GM. TEST MEAL

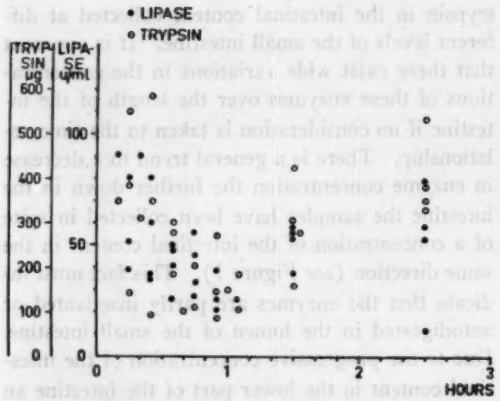

FIG. 7. ENZYME TIME RELATIONSHIP FOR TRYPSIN AND LIPASE IN THE INTESTINAL CONTENT FROM THE DUODENUM AND THE PROXIMAL JEJUNUM AFTER FEEDING 500-GM. TEST MEAL TO NORMAL HUMAN BEINGS

enzymatic content of the so-called *succus entericus* we have determined the invertase activity of the intestinal content at different levels. These values are given in Figure 8 and show a course very different from that of the pancreatic enzymes. There is very little invertase activity in the duodenum and the proximal jejunum where the pancreatic enzymes have their maximum values. The invertase activity varies greatly in spite of the fact that this enzyme is fairly stable *in vitro*, and has maximum values at a level about 200 cm. from the nose. If the activity of invertase and amylase in the intestinal content is compared it can be calculated that the amylase contained in 1 ml. intestinal content can digest around 5 Gm. starch per hour at 37° C. while the corresponding figure for the invertase activity is less than 0.02 Gm. saccharose. Thus there is a great difference in activity per ml. of intestinal content and it is also apparent from the absorption curve shown in Figure 3 that a disaccharide as lactose is absorbed at a level of the small intestine where the disaccharide splitting enzymes are absent or have a low concentration. It should be added that other experiments (41) show that the lactase activity of intestinal content follows the same course as the invertase activity and is only about half of that

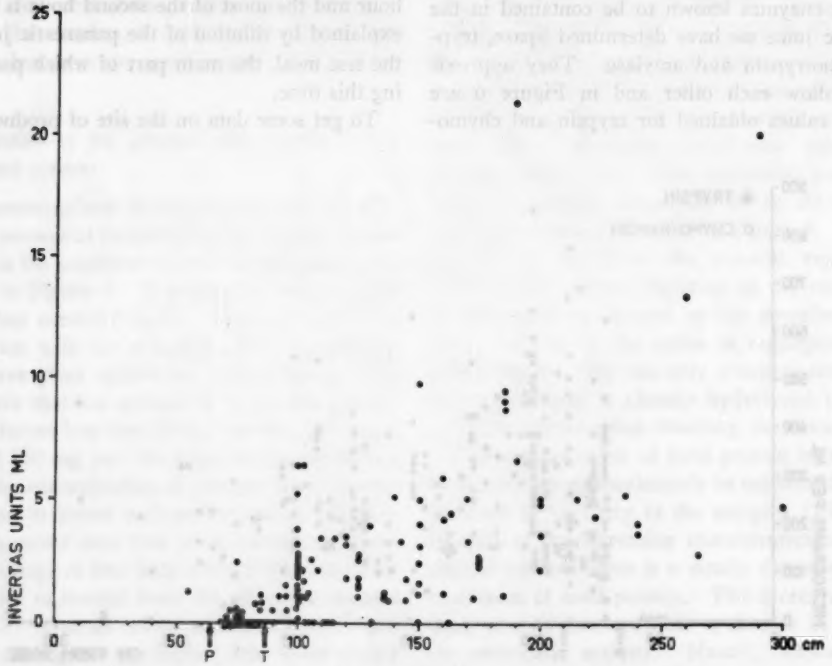

FIG. 8. CONCENTRATION OF INVERTASE OVER THE LENGTH OF THE HUMAN SMALL INTESTINE AFTER 500-GM. TEST MEAL

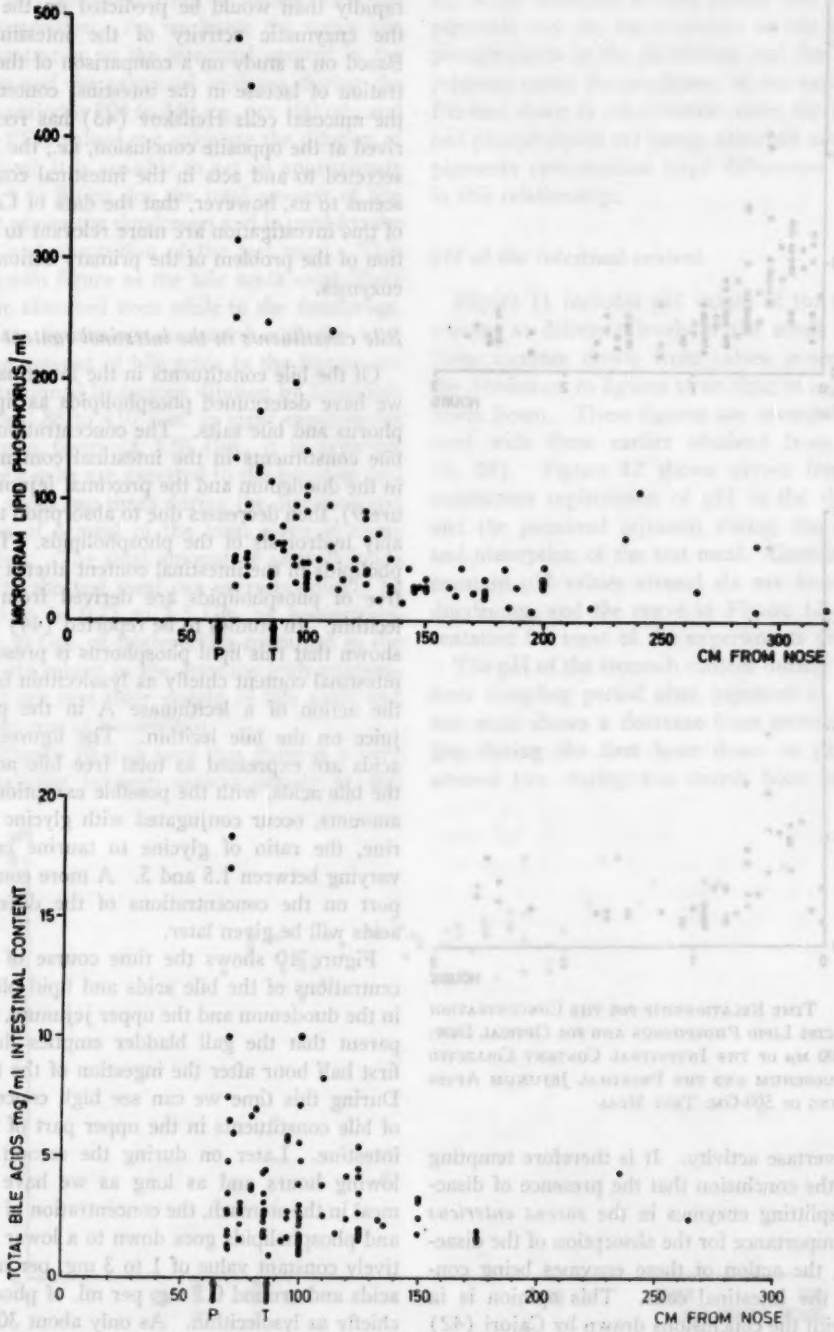

FIG. 9. CONCENTRATION OF TOTAL BILE ACIDS AND LIPID PHOSPHORUS IN THE INTESTINAL CONTENT AT DIFFERENT LEVELS AFTER FEEDING OF 500-GM. TEST MEAL

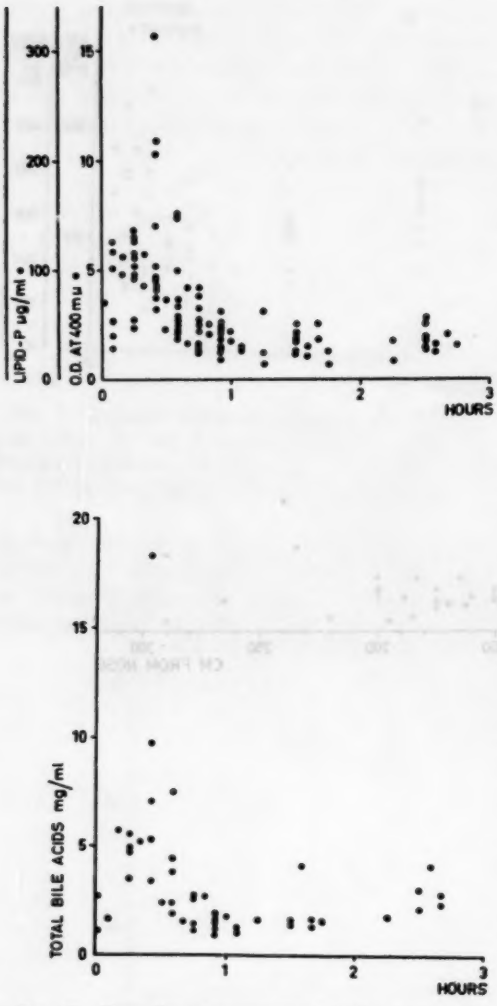

FIG. 10. TIME RELATIONSHIP FOR THE CONCENTRATION OF BILE ACIDS LIPID PHOSPHORUS AND FOR OPTICAL DENSITY AT 400 Mμ OF THE INTESTINAL CONTENT COLLECTED IN THE DUODENUM AND THE PROXIMAL JEJUNUM AFTER THE FEEDING OF 500-GM. TEST MEAL

of the invertase activity. It is therefore tempting to draw the conclusion that the presence of disaccharide splitting enzymes in the *succus entericus* is of no importance for the absorption of the disaccharides, the action of these enzymes being confined to the intestinal cells. This opinion is in accord with the conclusions drawn by Cajori (42) in experiments on intestinal loops of dogs that sucrose and lactose are absorbed much more rapidly than would be predicted on the basis of the enzymatic activity of the intestinal juice. Based on a study on a comparison of the concentration of lactase in the intestinal content and in the mucosal cells Heilskov (43) has recently arrived at the opposite conclusion, *i.e.*, the lactase is secreted to and acts in the intestinal content. It seems to us, however, that the data of Cajori and of this investigation are more relevant to the solution of the problem of the primary action of these enzymes.

Bile constituents in the intestinal content

Of the bile constituents in the intestinal content we have determined phospholipids as lipid phosphorus and bile salts. The concentration of these bile constituents in the intestinal content is high in the duodenum and the proximal jejunum (Figure 9), then decreases due to absorption and probably hydrolysis of the phospholipids. The phospholipids in the intestinal content after a test meal free of phospholipids are derived from the bile lecithin. In studies to be reported (44) it will be shown that this lipid phosphorus is present in the intestinal content chiefly as lysolecithin formed by the action of a lecithinase A in the pancreatic juice on the bile lecithin. The figures for bile acids are expressed as total free bile acids. All the bile acids, with the possible exception of trace amounts, occur conjugated with glycine and taurine, the ratio of glycine to taurine conjugates varying between 1.5 and 5. A more complete report on the concentrations of the different bile acids will be given later.

Figure 10 shows the time course of the concentrations of the bile acids and lipid phosphorus in the duodenum and the upper jejunum. It is apparent that the gall bladder empties during the first half hour after the ingestion of the test meal. During this time we can see high concentrations of bile constituents in the upper part of the small intestine. Later on during the second and following hours and as long as we have any test meal in the stomach, the concentration of bile acids and phospholipids goes down to a lower and relatively constant value of 1 to 3 mg. per ml. of bile acids and around 0.5 mg. per ml. of phospholipid, chiefly as lysolecithin. As only about 30 per cent of the test meal is passing the duodenum during the first hour it is apparent that most of the meal is

digested and absorbed from a milieu with the above composition. As we know the mean bile acid concentration in the intestinal content in the duodenum and the proximal jejunum during the digestion period is 204 to 330 mg. per 100 ml., and from the PEG values can calculate the dilution of the test meal, it is possible to get an approximate figure of 4 to 8 Gm. for the total amount of bile acids that passes the duodenum and is used for the digestion and absorption of the test meal. This is a minimum figure as the bile acids most likely start to be absorbed even while in the duodenum. When these figures are compared with the total circulating amount of bile acids in the human recently determined by isotope dilution by Lindstedt (45) to be 1.88 to 4.97 Gm. in eight experiments with a mean figure of 3.58 Gm., it is apparent that the bile salts are excreted to the intestine and absorbed about two times during the digestion and absorption of a meal of the kind given in these experiments. The total amount of phospholipids mixed with the test meal can correspondingly be calculated to be 0.75 to 1.5 Gm. as lysolecithin. Thus the 30 Gm. of triglycerides contained in the test meal are mixed in the intestine with approximately 20 per cent their weight of bile acids and 2.5 to 5 per cent of lysolecithin.

From Figure 10 it is seen that there is a very good parallelity between optical density at 400 mμ of the intestinal content mainly due to the bile pigments and the concentration of bile acids and phospholipids in the duodenum and the proximal jejunum under the conditions of our experiments. Further down in the intestine when the bile acids and phospholipids are partly absorbed and the bile pigments concentrated large differences are seen in this relationship.

pH of the intestinal content

Figure 11 includes pH values of the intestinal content at different levels of the small intestine. They increase slowly from values around six in the duodenum to figures at or close to eight in the lower ileum. These figures are essentially in accord with those earlier obtained from humans (6, 39). Figure 12 shows curves from direct continuous registration of pH in the duodenum and the proximal jejunum during the digestion and absorption of the test meal. Generally, very constant pH values around six are found in the duodenum, and the curve in Figure 12 is representative for most of the experiments performed.

The pH of the stomach content during the four-hour sampling period after ingestion of 500-Gm. test meal shows a decrease from around four to five during the first hour down to pH values around two during the fourth hour when the

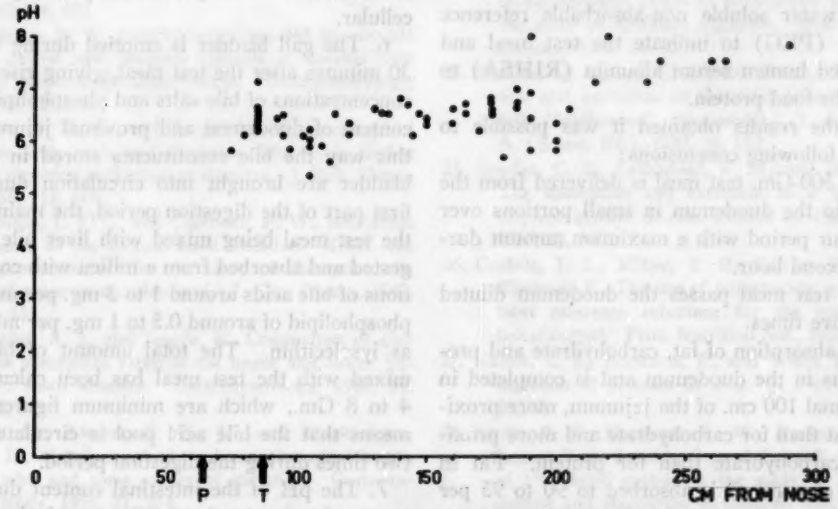

FIG. 11. pH OF THE INTESTINAL CONTENT COLLECTED AT DIFFERENT LEVELS OF THE HUMAN SMALL INTESTINE AFTER FEEDING OF 500-GM. TEST MEAL

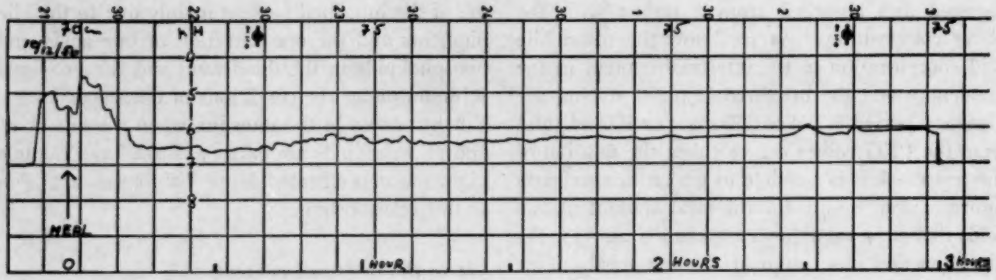

FIG. 12. CONTINUOUS REGISTRATION OF pH OF INTESTINAL CONTENT FLOWING FROM A TUBING DRAINING THE DUODENUM DURING DIGESTION OF A 500-GM. TEST MEAL

stomach starts to be empty. Apparently the proteins of the test meal effectively buffer the acid secretion of the stomach under the conditions of our experiments.

CONCLUSIONS

The intestinal digestion and absorption were studied in normal human beings, 16 to 40 years of age, subjected to intestinal intubation with a polyvinyl tubing (inner diameter, 2.1 mm.), allowing sampling of intestinal content at any level of the gastrointestinal tract.

The test subjects were fed a fluid test meal containing fat as corn oil, carbohydrate as glucose plus lactose, and protein as milk proteins in well-balanced proportions. The test meal also contained a water soluble non-absorbable reference substance (PEG) to indicate the test meal and I^{131}-labelled human serum albumin (RIHSA) to indicate the food protein.

From the results obtained it was possible to draw the following conclusions:

1. The 500-Gm. test meal is delivered from the stomach to the duodenum in small portions over a four-hour period with a maximum amount during the second hour.

2. The test meal passes the duodenum diluted three to five times.

3. The absorption of fat, carbohydrate and protein begins in the duodenum and is completed in the proximal 100 cm. of the jejunum, more proximal for fat than for carbohydrate and more proximal for carbohydrate than for protein. Fat in the form of corn oil is absorbed to 90 to 95 per cent and is not significantly diluted with endogenous fat in the small intestine; carbohydrate in the form of glucose plus lactose is absorbed to 100 per cent, and protein as indicated by RIHSA to 80 to 90 per cent.

4. The concentration in the intestinal content of enzymes contained in the pancreatic juice is highest in the duodenum and the proximal jejunum. Due to auto-digestion, the enzyme concentration decreases slowly in distal direction in spite of a progressive concentration in the same direction of the intestinal content. The result, however, is that a considerable concentration of enzymes is maintained over the length of the small intestine.

5. There seems to be reason to believe that the enzymes contained in the so-called *succus entericus* are of no or little importance for the intraluminar digestion and absorption. The major locus of action of these enzymes most probably is intracellular.

6. The gall bladder is emptied during the first 30 minutes after the test meal, giving rise to high concentrations of bile salts and phospholipid in the content of duodenum and proximal jejunum. In this way the bile constituents stored in the gall bladder are brought into circulation during the first part of the digestion period, the main part of the test meal being mixed with liver bile and digested and absorbed from a milieu with concentrations of bile acids around 1 to 3 mg. per ml. and of phospholipid of around 0.5 to 1 mg. per ml. chiefly as lysolecithin. The total amount of bile acids mixed with the test meal has been calculated to 4 to 8 Gm., which are minimum figures. This means that the bile acid pool is circulated about two times during the digestion period.

7. The pH of the intestinal content during digestion in the duodenum is around six and increases slowly in distal direction to values up to

eight. The pH in the duodenum and the jejunum is very constant during the digestion period.

ACKNOWLEDGMENT

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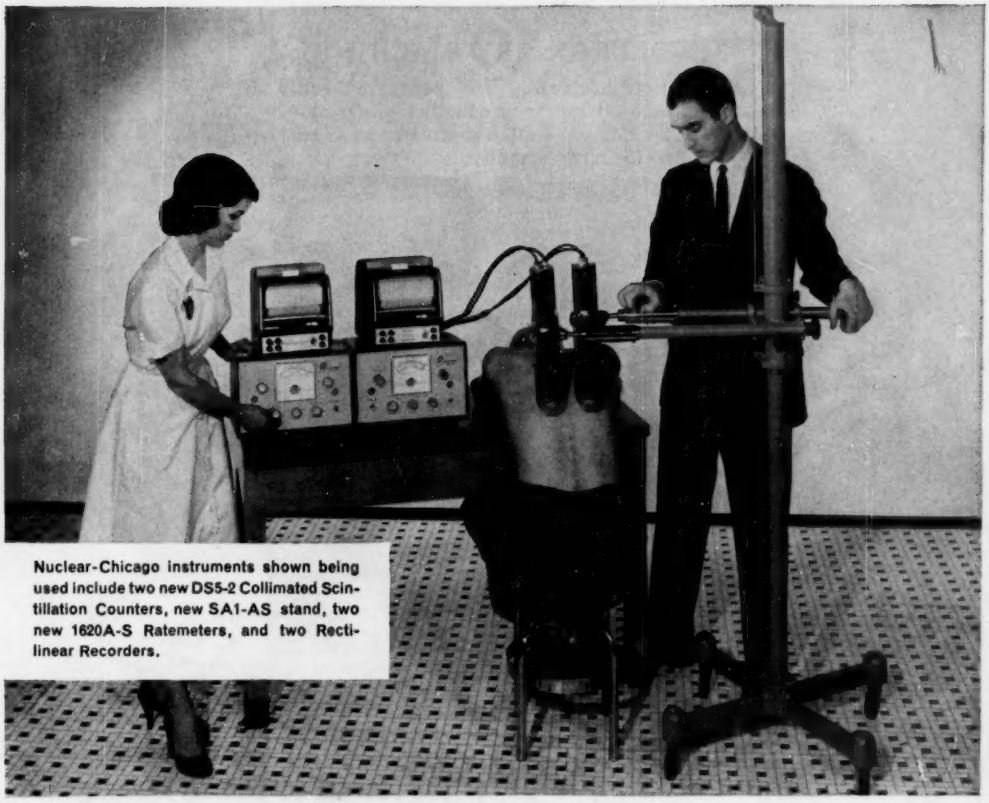

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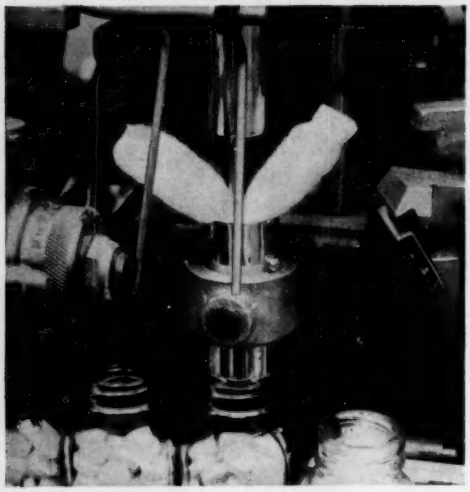

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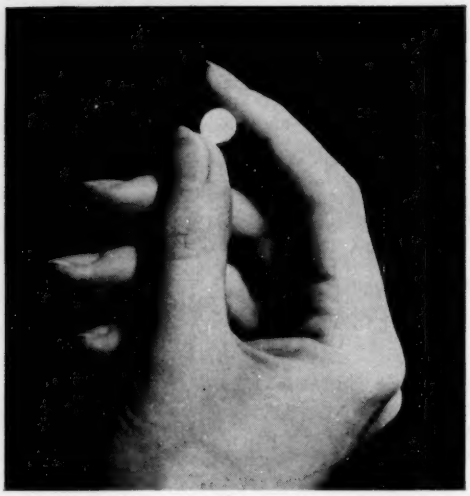

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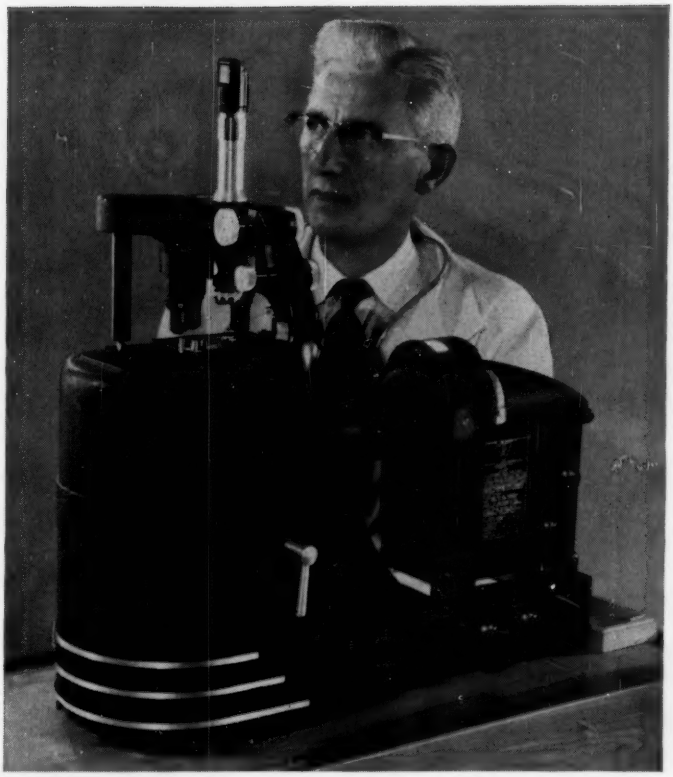

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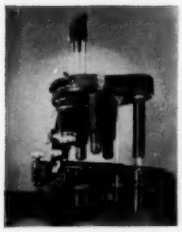

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